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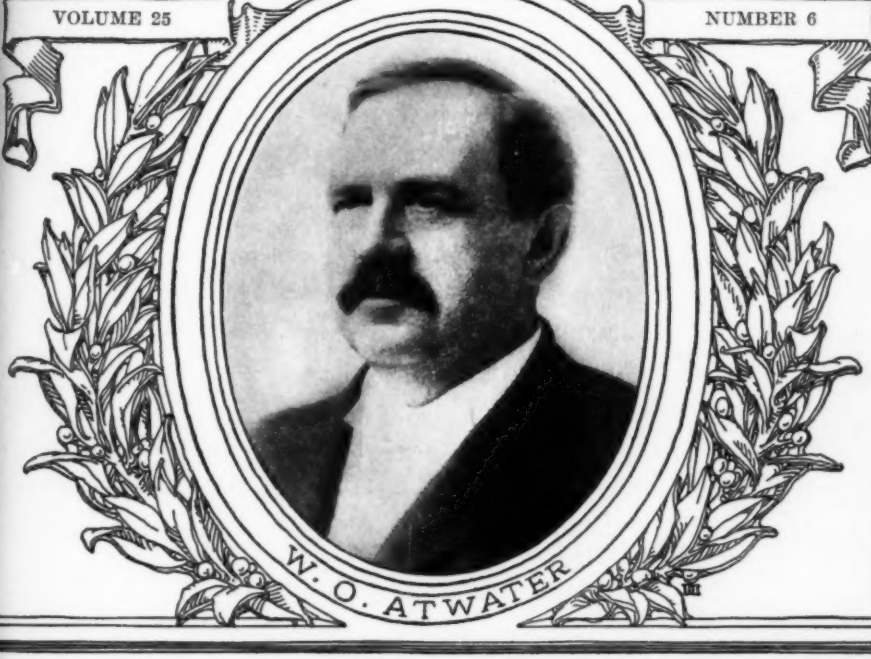

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WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA, PA.

THE EFFECTS OF INANITION AND RIBOFLAVIN DEFICIENCY UPON THE BLOOD PICTURE OF THE RAT[1]

CARROLL F. SHUKERS AND PAUL L. DAY
Department of Physiological Chemistry, School of Medicine, University of Arkansas, Little Rock

(Received for publication February 15, 1943)

This investigation is concerned with the effect on the blood picture in rats of inanition with adequate riboflavin intake as contrasted with the effect of riboflavin deficiency. The experiment was suggested by early observations from this laboratory that attempts to produce cataract in monkeys on diets somewhat similar to those which produce cataract in rats (Day, Langston and O'Brien, '31; Day and Langston, '34) resulted in leucopenia and anemia in monkeys (Day, Langston and Shukers, '35). In 1933 it seemed desirable to study the hematology of rats on cataract-producing diets. Distinct alteration in the blood picture of vitamin G-deficient rats was clearly shown but too few of the inanition controls survived for a sufficiently long period to clarify the role of inanition in this syndrome. After riboflavin deficiency was established as the factor responsible for keratitis and cataract (Day, Darby and Langston, '37) the experiment was repeated. In the 1940 experiments it was possible to maintain the inanition controls on experiment for as long a time as the deficient rats survived.

Citation of literature prior to 1936 or 1937 as evidence for the relation of riboflavin to hemopoiesis may be open to criti-

[1] Research paper no. 534, Journal Series, University of Arkansas. The authors acknowledge the assistance of Miss Virginia Mims and Mr. Jack Kolson in making some of the blood counts.

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cism since the vitamin B complex-deficient and vitamin G-deficient diets used were possibly deficient in factors other than riboflavin, and since the adjuvant added (yeast, liver or other substances as a source of riboflavin) also contained other components of the vitamin B complex. However, with this reservation in mind, the earlier reports are of interest. Jackson ('25) has reviewed the earlier literature and summarized the effects of inanition and malnutrition on the blood elements. Acute total inanition in rats produced a decrease in total leucocyte count associated with a relative neutrophil increase and a pronounced lymphocyte decrease. No significant changes occurred in the erythrocyte count. Although some authors found no significant change in the total or differential blood counts of rats on diets deficient in the vitamin B complex, others reported a leucopenia and a marked decrease in lymphocytes associated with a general atrophy of lymphoid tissue.

There was early evidence that inanition was a factor in the syndrome of vitamin G or riboflavin deficiency. Graham and Griffith ('33) found the utilization of food for growth to be decreased on diets low in vitamin G. Remp and Bing ('34) reported that inanition was a significant feature of the syndrome of vitamin G deficiency; the authors found that the number of erythrocytes and the hemoglobin concentration were slightly lower in vitamin G-deficient mice than in normal mice.

The data of Stucky and Brand ('33), von Drigalski ('35), and Smith and Stohlman ('34) did not indicate that vitamin G influenced erythropoiesis. Smith and Stohlman concluded that vitamin B_2 did not appear to be concerned with hemopoiesis in the albino rat. Oestreicher and Overbeek ('39) found no anemia but reported a prolongation of the physiological leucopenia that occurs in young rats on a diet deficient in part of the vitamin B complex but containing thiamine, riboflavin, and nicotinic acid.

On the other hand, several investigators have obtained data which they interpret as indicating that riboflavin does affect hemopoiesis. Sure, Kik and Smith ('31) reported that

three out of sixteen rats without dermatitis had an anemia on a vitamin G-deficient diet, while ten out of fourteen rats with dermatitis on the same diet were anemic. No significant changes were found in the total and differential leucocyte counts. Guha and Mapson ('31) and Kohls ('32) found an anemia in vitamin G-deficient rats. The latter author also reported a moderate degree of leucopenia with absolute lymphopenia and a relative leucocytosis. The addition of autoclaved yeast gradually restored the blood to normal.

The observation that feeding sulfanilylguanidine or succinylsulfathiazole to rats results in a leucopenia (Spicer, Daft, Sebrell and Ashburn, '42) arouses renewed interest in the effect of dietary factors upon the blood picture of rats.

EXPERIMENTAL

The conditions of the experiment were identical with those previously described for the production of cataract (Day, Langston and O'Brien, '31; Day and Langston, '34). Stock albino and pied rats 21 days old were divided into three groups of litter mate controls and kept under identical conditions except for their diets. The rats were housed in separate cages with raised wire bottoms. In the 1933 experiments the deficient rats received diet 625 (Day and Langston, '34) which had the following percentage composition: alcohol-extracted casein, 18; Osborne and Mendel salt mixture ('19), 4; butterfat, 8; cod liver oil, 2; cornstarch, 43; and cornstarch carrying an 80% alcohol extract of rice polish, 25. A second group (normal controls) received a diet of the same composition except that 20% of the diet was dried brewers' yeast, this replacing an equivalent weight of starch. The third group (inanition controls) received the same diet as the normal controls but the food intake was restricted.

In the 1940 experiments all groups received a diet identical with the deficient diet (no. 625) described above except for two minor modifications: the casein was a commercially available vitamin-free preparation,[2] and the salt mixture was the

[2] "Labco" brand, manufactured by the Labco Products Department of the Borden Company.

one described by Hubbell, Mendel and Wakeman ('37). In this series the normal controls received 120 μg. of riboflavin per rat twice each week and unrestricted amounts of basal diet. The inanition animals likewise received 120 μg. of riboflavin per rat twice each week but the basal diet intake was restricted to amounts approximately equal to those eaten by the deficient animals.

Growth data previously reported (Day, Darby and Langston, '37), the growth data given herein, and unpublished experiments indicate that the basal diets used are capable of supporting good growth when supplemented with adequate amounts of riboflavin. It can therefore be assumed that the basal diet is not deficient in other substances required by the rat.

Blood was obtained from the tail vein. Erythrocyte, leucocyte, and differential counts were made by standard technics. Bureau of Standards certified pipettes and counting chambers were used. Hemoglobin was determined by the Newcomer method.

The data of the 1933 experiments (table 1) indicate a leucopenia in the inanition and riboflavin-deficient animals, much more marked in the former. There was a relative increase in neutrophils and a corresponding decrease in lymphocytes. The mean levels of hemoglobin, erythrocytes, monocytes, and eosinophils for all experiments were not significantly affected. The mean total leucocyte counts of the three groups for the entire experiment were 13,200, 4800, and 10,400 per cubic millimeter in the normal, inanition and riboflavin-deficient rats, respectively. The mean percentages of neutrophils in the same groups were 16 (2110 per cubic millimeter), 29 (1390 per cubic millimeter) and 28 (2920 per cubic millimeter). There was a relative and absolute decrease in lymphocytes from 80% (10,500 per cubic millimeter) in the normals to 68% (3260 per cubic millimeter) and 69% (7200 per cubic millimeter) in the inanition and deficient rats, respectively.

Although the data justify the foregoing general conclusions, study of the individual data reveals several exceptions which deserve comment. Two of the seven deficient animals had an anemia with erythrocyte counts of 3.21 and 1.88 millions per cubic millimeter, respectively, on the sixty-fifth day of experiment. None of the normal or inanition animals revealed any disturbance in erythropoiesis. Total leucocyte counts in inanition animals were under 7000 per cubic millimeter after the thirteenth day; six of eleven counts were under 4000 per cubic millimeter. However, only half of the inanition controls had a marked increase in the percentage of neutrophils. All leucocyte counts on normal animals were over 7000 per cubic millimeter and only three out of twenty-seven counts were under 11,000 per cubic millimeter. Total leucocyte counts in the riboflavin-deficient animals were quite variable. Two of the seven rats did not show a leucopenia but one of these had trypanosomiasis. Omitting the data of these two animals, only three total leucocyte counts were over 11,000 per cubic millimeter in 116 days on diet. Two of the seven deficient animals did not have a marked increase in the percentage of neutrophils.

Ocular manifestations were not found in any of the normal animals but keratitis was observed in one of the inanition controls on the thirtieth day. This cleared up completely later. One of the riboflavin-deficient animals in the 1933 experiments died on the thirty-seventh day without eye changes. Keratitis and later cataract were seen in the remaining six animals between the twenty-sixth and sixty-third days on diet. White blood cell changes preceded the ocular signs.

A different strain of rats was used in the 1940 experiments. In this series no ocular lesions were seen in any of the normal or inanition animals. An ophthalmia and later cataract were observed in all of the riboflavin-deficient rats between the thirty-second and fifty-eighth days. Blood cell changes preceded the eye signs. The normal total leucocyte count in these animals was less than half that found in the animals used in the 1933 experiments. From the fifth to the eighteenth

TABLE 1

Effects of inanition and riboflavin deficiency on the blood picture of rats.

TIME	DIET	NO. OF RATS	NO. OF COUNTS	MEAN TOTAL GAIN IN WEIGHT	MEAN WEEKLY FOOD INTAKE	HEMOGLOBIN PER 100 ML.	ERYTHROCYTES	LEUCOCYTES	NEUTROPHILS		LYMPHOCYTES	
days				*gm.*	*gm.*	*gm.*	*millions per c.mm.*	*per c.mm.*	*per cent*	*per c.mm.*	*per cent*	*per c.mm.*
1933 experiments												
0–14	Normal	1	1	34		11.29	4.39	7200	21	1510	73	5250
	Inanition	3	3	1	21	12.34	5.39	7500	28	2100	70	5250
	Deficient	4	4	14		15.02	5.84	7300	16	1170	82	5970
15–28	Normal	4	5	54		12.65	5.81	11900	19	2260	78	9300
	Inanition	3	4	4.5	21	14.76	7.55	3900	33	1290	66	2580
	Deficient	5	7	15		12.68	6.31	11200	33	3700	65	7280
29–42	Normal	3	6	102		14.92	6.22	14700	16	2350	80	11750
	Inanition	3	4	14	25	14.65	6.76	4300	32	1370	64	2750
	Deficient	4	6	14		13.02	6.63	11700	34	3980	65	7600
43–56	Normal	3	4	151		15.80	6.30	14300	12	1710	84	12000
	Inanition	1	1	17	28	19.84	6.12	2900	15	435	81	2350
	Deficient	2	2	12		10.90	4.65	9200	33	3040	64	5880
57–70	Normal	3	5	166		16.22	7.45	15400	21	3240	76	11700
	Deficient	3	3	12		7.27	3.89	8900	26	2320	70	6240
71–84	Normal	2	4	198		15.18	7.40	17700	16	2840	81	14350
	Deficient	1	1	25		15.60	8.95	11600	25	2900	69	8000
85–116	Normal	1	2	252		14.56	6.85	21300	21	4470	78	16600
	Deficient	2	7	20		12.81	5.05	9300	40	3820	55	5100
117–172	Deficient	2	7	32		15.53	6.55	12400	48	5950	47	5820

Summary of all counts — 1933 experiments												
10–94	Normal	5	27			14.19	6.12	13200	16	2110	80	10500
8–44	Inanition	4	12			14.50	6.72	4800	29	1390	68	3260
9–172	Deficient	7	37			13.42	6.29	10400	28	2920	69	7200
1940 experiments												
0–14	Normal	2	2	4	25	9.8	4.7	4500	30	1350	67	3000
	Inanition	10	10	2	21	10.5	5.2	4400	23	1010	76	3340
	Deficient	2	2	2	22	11.1	6.1	4000	17	680	82	3300
15–28	Normal	4	4	34	36	15.0	5.5	3900	19	750	81	3150
	Inanition	11	12	7	18	13.3	6.4	3100	31	980	63	1950
	Deficient	3	3	−3	19	16.3	5.8	5700	42	2390	56	3200
29–42	Normal	4	8	45	37	15.3	6.1	5900	24	1420	74	4380
	Inanition	11	20	5	18	12.9	5.3	2500	30	750	70	1750
	Deficient	3	6	−2	18	15.1	6.6	2800	51	1430	49	1370
43–56	Normal	4	5	61	51	15.9	6.0	6200	31	1920	66	4080
	Inanition	8	13	10	16	13.0	6.1	2600	27	720	72	1870
	Deficient	5	6	−1	17	14.8	5.9	4300	46	1980	53	2280
57–70	Normal	4	8	107	64	15.0	6.3	5600	22	1230	76	4260
	Inanition	7	12	11	18	13.5	6.3	2900	40	1160	59	1710
	Deficient	4	7	−1	16	15.3	6.5	3300	58	1910	41	1350
71–84	Normal	4	8	128	55	14.3	6.5	8100	24	1950	73	5920
	Inanition	7	13	15	16	13.0	6.1	2200	30	660	69	1520
	Deficient	4	5	0	16	13.8	6.1	2800	51	1425	48	1345
85–125	Normal	4	13	148	51	14.0	6.0	7300	18	1320	77	5630
	Inanition	5	12	13	14	12.9	5.9	2300	59	1360	40	920
	Deficient	3	5	1	16	13.6	6.1	3300	61	2075	36	1180

week the total leucocyte counts were consistently lower in the inanition and riboflavin-deficient rats than in the normal animals (table 1). In general the counts were lowest in the inanition controls. Three of the eleven inanition controls had no pronounced increase in percentage of neutrophils. As a result of this and the uniformly low total leucocyte counts, an absolute increase in mean number of neutrophils was seen in only one period. In only four out of seven periods was an absolute increase in number of neutrophils found in the riboflavin-deficient animals. The only evidence of disturbance in erythropoiesis was a slight reduction in hemoglobin and erythrocytes in one riboflavin-deficient rat near the close of the experiment. There was a relative and an absolute reduction in lymphocytes. This is in accord with the observations of others on acute inanition (Jackson, '25) and vitamin G deficiency (Kohls, '32) and is in agreement with the results of our 1933 experiments.

SUMMARY

Albino and pied rats, 21 days of age, were divided into three groups. One group was given free access to a diet deficient in riboflavin but adequate for good growth with regard to all other nutritive factors. A second group (normal controls) was given the same diet ad libitum supplemented with 120 μg. of riboflavin twice weekly per rat. A third group (inanition controls) was also given 120 μg. of riboflavin twice weekly per rat but the basal diet intake was restricted to amounts approximately equal to those eaten by the deficient animals. Hemoglobin determinations, total erythrocyte and leucocyte counts, and differential counts were made at frequent intervals during the experiment.

The deficient animals exhibited a leucopenia with a relative and absolute decrease in lymphocytes. A relative increase in neutrophils was evident by the third or fourth week on diet. The changes in leucocytes were seen prior to changes in the lens and cornea of the eye. An anemia was found in several of the deficient animals toward the end of the experiment.

Similar changes were found in the number and distribution of leucocytes in the inanition controls; total leucocyte counts were usually lower in these than in the deficient animals. No cataract was observed in the inanition group, and a transient keratitis was seen in only one animal.

These data indicate that the changes in number and distribution of leucocytes in rats receiving a riboflavin-deficient diet are probably the result of inanition.

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THE ASSAY OF VITAMIN B_1 IN NEW ZEALAND MATERIALS BY THE THIOCHROME METHOD

DORIS I. ALLEN

Departments of Physiology, Medical School, and Home Science, University of Otago, New Zealand

(Received for publication January 13, 1943)

Particularly at the present time with impending limitations in the country's diet, a more extensive knowledge of the distribution of vitamin B_1 in food and biological materials is of immediate national importance and concern. No rapid chemical method for the estimation of this vitamin had been employed in New Zealand. This study was undertaken primarily to establish such a method. After studying the merits of the diazo, fermentation, and thiochrome techniques, the latter was chosen as being the simplest and most reliable.

This paper reports (1) the results obtained by application of the method to New Zealand cereal products and (2) the assessment of the level of vitamin B_1 nutrition of normal subjects from their urinary excretion values.

GENERAL METHODS

Pyke has been the notable worker in developing Jansen's ('36) original thiochrome method and in employing it for the estimation of vitamin B_1 in food materials. Using his technique (Pyke, '39) as modified by Booth ('40) and combined with the suggestions of other workers, a method was evolved, tested for accuracy, and applied to a number of New Zealand flours and breads.[1]

[1] The data in this section are taken from the thesis presented by the author to the Faculty of Home Science, University of Otago, in partial fulfillment of the requirement for the M. H. Sc. Degree, November, 1941. Further details will be found in the thesis.

For the assays on breakfast foods and urine the working details were simplified by adopting the process recommended by Harris and Wang ('41 a, b),[2] with a few minor changes. On the evidence presented by Conner and Straub ('41) and McFarlane and Chapman ('41), a 45% solution of NaOH was used instead of a 30% solution, with 2 cc. of the unknown and 2 cc. of methanol. It was found advantageous to add the ethanol for clarification before removal of the isobutyl layer from the aqueous layer. One milliliter only of 96% ethanol was used. Nitrogen stirring was employed to prevent the oxidative action of the air which may cause oxidation of the vitamin in the "blank" tube.

The standard solution of vitamin B_1 was prepared from pure thiamine hydrochloride diluted with an acetate buffer of pH 4.0, toluol (0.2 ml. per 100 ml.) added and the solution stored in a refrigerator. The toluol had no effect on the resulting fluorescence. Checking the solution against fresh preparations, it seemed advisable to prepare a new standard at least every 2 months. The standard solution of thiochrome used for matching was made up in a 10 ml. volumetric flask, using the isobutyl extract from the "blank" tube with the addition of an accurately measured amount of the isobutyl extract from the "control" tube (which contained the thiochrome from the oxidized standard solution of vitamin B_1) to make the resulting fluorescence approximately equal to that of the "unknown." Double quantities were used in the "blank" tube so that sufficient isobutyl extract was available for the preparation of a trial standard. This was essential with the urine samples whose vitamin B_1 values were more variable than those of the food materials.

Unless the technician was well experienced it was considered that the simple visual method of comparison adopted by Wang and Harris ('39) allowed for too large a personal error in judgment. Therefore an improved technique, described in more detail below, was evolved.

[2] This study was completed before the publication by Wang and Harris ('42) giving further notes on their technique.

Jansen ('36) found the fluorescence of the thiochrome to be more intense in isobutyl alcohol than in water. He gave no further reason for using isobutanol, and subsequent workers have followed his suggestion. All the assays reported here were extracted with this solvent. However, as only a limited supply of the alcohol was available, normal and secondary butyl alcohols were tested to see if they would prove possible substitutes. Farrer ('41) stated that the normal form is satisfactory; this was confirmed. The secondary alcohol did not appear suitable, much lower results being obtained.

Apparatus for measurement.

A colorimeter of the Duboscq type [3] with nephelometer attachments was employed for the fluorescent matching. The tube containing the standard solution of thiochrome was always placed on the same side and the movable jacket on this tube was set at a convenient point. The comparisons were made by adjusting the jacket on the tube containing the unknown solution until the images showed equal illumination. Six readings were made alternately from above and below, and the average taken. To estimate the source of error during matching, a series of determinations were made with the same solution on each side. The variation was about one scale division, the average error being 1.8% with a range of $\pm 5\%$.

A 125-watt ultra violet globe was employed as the exciting source. The outer envelope of ordinary frosted glass was removed because it did not allow enough of the ultra violet rays to pass through. Visible light from the globe was cut out by a filter of Chance's Uviol glass, similar in composition to Wood's glass used by other investigators. The globe was mounted in a blackened tin box and the total height of the apparatus adjusted so that the filter glass window was directly opposite the tubes holding the fluorescent material. A shutter allowed the ultra violet light to be screened off when necessary. Matching could be commenced within 7 min-

[3] Made by Bausch and Lomb.

utes after entering the dark room, red glasses being worn while reading the scale in order not to spoil the adaptation.

VITAMIN B_1 CONTENT OF NEW ZEALAND CEREAL PRODUCTS

Results

Flours and breads. Table 1 gives the results of analyses on New Zealand flours and breads, using Pyke's ('39) method as modified by Booth ('40).

TABLE 1

Vitamin B_1 content of flours and breads.

SAMPLE	NO. OF DETERMINATIONS	AVERAGE RECOVERY OF ADDED VIT. B_1	VITAMIN B_1 µG./GM.	
			Dried product	Fresh product
		%		
New Zealand whole meal flour [1]	5 [2]	107		4.30
New Zealand white flour [3]	2	96		2.00
Dunedin whole meal flour	2	100		5.27
Whole meal bread, 100%	3	118	3.71	2.61
Bread prepared from 80% extraction flour	4 [4]	107	3.44	2.37
Bread prepared from 70% extraction flour	3	105	1.87	1.28

[1] Prepared from a composite wheat mixture consisting of the wheat grists from all the New Zealand mills in proportion to the amount made by each mill.

[2] Standard deviation from the mean .048 µg., probable error 3.2%.

[3] A composite consisting of nearly all the white flours milled in New Zealand in proportion to the amount made by each mill.

[4] Standard deviation from the mean .058 µg., probable error 3.9%.

Breakfast foods.[4] The breakfast foods tested were obtained from the open market. A representative sample of each packet was ground finely and the assays carried out in duplicate on weighed portions. In the majority of cases the duplicate results agreed within 0–10%. If any varied by more than 15%, further assays were made.

The results of the determinations on these foods are given in table 2. For comparison they have been divided into two

[4] The author is indebted to the staff of the New Zealand Nutrition Research Department for technical assistance in preparing the breakfast cereals for assay.

series. Included under series I are those products sold as "ready-to-eat" cereals which have been sufficiently precooked to require no further treatment by the consumer; under series II are the uncooked and partially precooked cereals.

No attempt was made to determine the range of values for a particular brand. The determinations were made on one

TABLE 2

Vitamin B_1 content of breakfast foods.

NUMBER	DESCRIPTION	MANUFACTURER	VITAMIN B_1 µg./gm.
	Series I — Precooked, ready-to-eat		
1	Biscuit, flaked wheat, malted	A	1.15
2	Biscuit, flaked wheat, malted	B	1.01
3	Biscuit, flaked wheat	A	1.06
4	Flaked rice	C	0.47
5	Flaked wheat	C	Negligible
6	Puffed rice	A	Negligible
7	Puffed wheat	A	Negligible
8	Bran product	A	2.29
9	Wheat product for infant feeding. Precooked and fortified.	D	4.21
	Series II — Uncooked, or partially precooked		
Oat products			
10	Oatmeal — fine	E	5.23
11	Oatmeal — fine — partially precooked	F	5.13
12	Rolled oats	F	4.55
13	Rolled oats	G	4.32
14	Oatmeal — coarse	H	4.29
15	Oatmeal — partially precooked	H	4.05
16	Rolled oats — partially precooked	I	3.94
17	Rolled oats	H	3.55
18	Oatmeal — partially precooked	H	2.77
19	Oatmeal — fine	H	2.47
Wheat products			
20	Wheatmeal — 100%	B	4.32
21	Wheat product. Some of the coarser bran removed and small amount of wheat germ added.	B	4.13
22	Wheat product — semolina	H	1.81
Miscellaneous			
23	Fine meal of wheat, maize and rice	J	5.01
24	Bran flakes	H	3.55
25	Barley product	E	0.99

packet only of each product. As there will be some variation from packet to packet according to age, storage conditions, and the vitamin B_1 content of the harvested grain, depending on the crop and the season, the value given for a particular product should not be considered as definitely fixed.

Those products listed under series II are generally cooked and served as porridge. The determinations were made directly on the cereals, but the figures given may be used to calculate the value of the cooked products because the ordinary household method of cooking porridge destroys little, if any, of the vitamin B_1 (Lane et al., '42; Slater and Rial, '42; Hanning, '41).

DISCUSSION

Flours and breads. An average New Zealand wheat was found to have a vitamin B_1 content of 4.3 μg. per gram, the same value being obtained by Slater and Rial ('41). It appears that the New Zealand wheats resemble the English in B_1 content, and equal in value only the poorest of the Australian and Canadian wheats. The average white flour value was 2.0 μg. per gram. On an equivalent weight basis, the white flour contains 46.5% as much vitamin as the whole wheat.

The closer the milling is carried to the bran layers, the higher will be the vitamin B_1 content of the resultant flour. This is shown by the results obtained from the breads made from 70, 80, and 100% extraction flours with values of 1.28, 2.37, and 2.61 μg. per gram, respectively. Copping and Roscoe ('37) have shown that there is no appreciable loss of vitamin B_1 during the baking of a loaf, while these workers and Baker, Wright and Drummond ('37) have proved that the yeast added contributes very little to the total vitamin B_1 content of the bread. Therefore the values obtained for yeast breads correspond very closely to the B_1 value of the particular flour used. The vitamin B_1 contents of the 70 and 80% extraction flours from which the breads were made, allowing for the additional water, are 1.87 and 3.44 μg. per gram, respectively; or 30.4 and 64%, respectively, of the original 4.3 μg. contained in the whole grain.

Breakfast foods. Ease of preparation has caused increased popularity of "ready-to-eat" and partially precooked cereals. Because of the laxity of the advertising laws, the New Zealand public is led to believe that these products contain the full vitamin content of the original grain, but such claims are without any experimental basis. Knowing that in processing drastic heat treatment is used, these extravagant statements were doubted. Few figures are available for the vitamin B_1 content of such foods; therefore analyses were made of the products on the New Zealand market.[5]

The "ready-to-eat" breakfast cereals studied (with the exceptions of no. 8, the prepared bran, and no. 9, the fortified cereal — see table 2), cannot be considered as important dietary sources of vitamin B_1. They should not be classified as "whole" cereals; indeed some have lost all their vitamin B_1. The "ready-to-eat" bran, no. 8, in the form of fine, crisp particles, was more appetizing than the untreated product, no. 24, but significantly lower in vitamin B_1.

The oat products ranged from excellent to good sources of vitamin B_1. There was some variation shown but it is attributed to the different oat crops used and to the different manufacturing processes; for instance, the oatmeal and rolled oats highest in vitamin B_1 came from the same mill "F"; products from mill "H" were consistently lower. The value for no. 19 was below the average for the oatmeals yet there was no statement made that it was precooked. The determinations were repeated on a new packet but the same low results were obtained.

There is no outstanding difference in the vitamin B_1 value of those products stated by the manufacturer as being either partially precooked or those which were apparently untreated. It appeared, however, that many of the rolled oats and oatmeals must have been subjected to steam treatment, although no mention was made of this, because the time specified for cooking was often low, suggesting that some preheating process had been applied.

[5] Since this work was completed a similar study has been reported by Slater and Rial ('42) working on Australian products.

The 100% whole meal breakfast cereal was not as rich as the best oat product, but was above the average; its value of 4.32 μg. per gram was in good agreement with the 4.30 μg. per gram given in table 1 for the average New Zealand whole meal. White flour and semolina are both prepared from the endosperm of the wheat grain. The value obtained for the semolina breakfast cereal was 1.81 μg. per gram, considerably lower than that of the 100% product, and close to the value of 2.0 μg. per gram found for the white flour (table 1).

Whole cereals rank high among the chief dietary contributors of vitamin B_1; they supply one-quarter of the total in the average American diet (Lane et al., '42).

On comparing the figures given in table 2 for series I (omitting nos. 8 and 9), and series II, it is evident that if the public substitutes a "ready-to-eat" cereal for its oat or whole wheat porridge it is unwittingly replacing a vitamin B_1 rich dish with a vitamin B_1 free or poor material.

ASSESSMENT OF THE LEVEL OF VITAMIN B_1 NUTRITION FROM URINARY EXCRETION VALUES

It is generally agreed that the amount of vitamin B_1 appearing in a 24-hour urine sample is a definite function of the amount of the vitamin habitually ingested. On the basis of recent investigations, standards have been suggested for the interpretation of the urinary vitamin B_1 value in terms of nutritional status.

This section reports assays carried out on urinary samples from normal male subjects and the application of these standards to assess the adequacy of their daily vitamin B_1 intake.

Experimental

The subjects were thirty-nine normal, apparently healthy, male medical students, with similar daily routines. Their average age was approximately 21 years. Of these, nine resided in their own homes, eighteen in University colleges, and twelve in different lodgings. The study was conducted over several weeks during the winter term. On the day the

urine sample was collected the subjects subsisted on their usual diets. Neither the weight of the food nor its vitamin B_1 content was known. However, during the collection period all food consumed with approximate amounts was noted and this information indicated the type of diet taken.

Collection of urine. From each subject one 24-hour specimen of urine was collected in a dark bottle containing a few drops of toluol and 5 ml. of glacial acetic acid which was sufficient to bring the pH to approximately 4. Urine so treated, according to Stockholm et al. ('41) retains its vitamin B_1 indefinitely.

On rising the subject emptied his bladder as completely as possible. This sample was discarded. The collection began from the next sample excreted for the following 24 hours. The total quantity was measured, a representative sample taken and stored in a refrigerator. The assays were carried out in duplicate on the same day. The results generally agreed within 10%; if not, further determinations were made.

Test dose procedure. As yet there is no agreement as to either the size of the test dose or the mode of administration. One milligram of thiamine hydrochloride was considered sufficiently large and near to the physiological requirement. Oral administration was chosen since it was the simplest. The dose was taken in tablet form immediately after the midday meal, as recommended by Melnick et al. ('39, '41), and Jowett ('40).

Experimental method. The assays were made by Harris and Wang's ('41 a, b) method with the modifications mentioned above. A preliminary trial, as described by these authors ('39), to determine the optimum amount of $K_3Fe(CN)_6$ to add to the unknown was carried out on each urine sample.

Results

The urinary volumes bore no relationship to the vitamin B_1 excreted as they were within the normal limits, the range being 700–2500 cc. (Marrack and Höllering, '39; Borson, '40).

In view of the reports by Wang and Yudkin ('40), and Cahill ('41), no concern was given to the fat-carbohydrate ratio of the diets consumed during the test periods. The 24-hour value is a function of the body's reserves as well as of the diet during collection. Each subject had been in his particular residence for some months before the assays were made; thus, his reserves were stabilized at that point of saturation permitted by his diet. The urinary excretion under the conditions of the test has therefore been accepted as being a sensitive index of the adequacy of the diets as regards vitamin B_1.

TABLE 3

Urinary excretion of thiamine.

RESIDENCE AND SUBJECT	URINARY THIAMINE	RESIDENCE AND SUBJECT	URINARY THIAMINE	RESIDENCE AND SUBJECT	URINARY THIAMINE	RESIDENCE AND SUBJECT	URINARY THIAMINE
	µg./24 hr.		*µg./24 hr.*		*µg./24 hr.*		*µg./24 hr.*
College A		*College B*		*Home*		*Lodgings*	
3	205	23	174	19	441	10	158
1	200	30	166	12	202	47	122
13	195	53	153	31	193	2	107
33	189	40	147	26	181	11	98
14	183	9	119	32	146	39	94
15	162	Average	152	25	145	52	94
16	121			56	141	48	89
34	121			21	133	24	79
4	119	*College C*		44	107	46	79
42	108	43	192	Average	188	45	74
41	103	38	175	Average (omitting 19)	156	27	55
Average	155	Average	184			55	43
Average for all colleges = 157						Average	90

The excretions of the thirty-nine subjects are listed in table 3 according to residence; they vary from 43 to 441 µg. per 24 hours, the average range being 100–200 µg. Good agreement is shown between the excretions of those subjects residing in the same college. The slight differences are attributed partly to individual variation and partly to the difference in the vitamin B_1 content of the food served on the day of collection. That the individual variation is not of great importance is shown by the excretions of several pairs of

TABLE 4

Urinary excretion of vitamin B_1 before and after a test dose of 1 mg.

SUBJECT	RESIDENCE	EXCRETION		EXTRA EXCRETION AFTER TEST DOSE	
		Before test dose	After test dose		
		µg./24 hr.	*µg./24 hr.*	*µg.*	*% test dose*
19	Home	441	571	130	13
15	College A	162	254	92	9
26	Home	181	271	90	9
27	Lodgings	55	96	41	4
55	Lodgings	43	69	26	3

TABLE 5

Urinary vitamin B_1 excretion values found by the thiochrome method.

AUTHOR	RANGE	MEAN	CONCLUSIONS AND COMMENTS
	µg./24 hr.	*µg./24 hr.*	
Benson et al. ('41)	96–602	268	22 normal children — diet supplied contained 999 µg.
Borson ('40)	75–290	166	8 normal males.
Carden et al. ('40)	108–390		11 normal males — normal excretion considered to be at least 100 µg.
	5–70		9 patients suspected of vitamin B_1 deficiency.
Hilderbrandt ('40)	80–450		Non-pregnant women; usual range 120–200 µg.
			During pregnancy less than 80 µg.
Hills ('39)	50–170	100	6 normal subjects.
Jolliffe et al. ('39)	319–676		5 normal males. *Control period:* Intake 2.44–2.63 mg., 13–26% excreted.
	35–108		*Deficiency period:* Intake 0.473 mg., 7–23% excreted.
	339–806		*Recovery period:* Intake 2.63–3.83 mg., 12–21% excreted.
Jowett ('40)	39–144		4 normal males.
Mason and Williams ('42)	90–152	119	5 females. Diet supplied 800 µg. or 400 µg. per 1000 calories. Concluded to be sufficient for minimal needs if 100 ± 10 µg. excreted when intake on test day about 1000 µg.
Wang and Harris ('39)	90–480		Usual range 150–240 µg. Suggested below 90 µg. and delayed response to a test dose indicates deficiency.
Wang and Yudkin ('40)	137–233		4 normal males — diet supplied contained 900 µg.

students, each pair identical as to residence, day of collection, type and amount of food consumed. Subjects 41 and 42 excreted 103 and 108 μg., subjects 13 and 14, 195 and 183 μg., and subjects 45 and 46, 74 and 79 μg., respectively.

Because of his outstandingly high excretion the collection from subject 19 was repeated after a 6-week period, but the same high level was obtained. His diet was known to be excellent; for years he had eaten only homemade 100% whole meal bread. He excreted, also, the highest percentage of the test dose, indicating that his tissue stores were well saturated.

The results obtained after the administration of the test doses are given in table 4. Table 5 gives a summary of the 24-hour excretion values obtained by other investigators using the thiochrome technique.

Discussion

The average of the 24-hour excretions for the eighteen college students was 157 μg., virtually equal to that of the home subjects, 156 μg. (omitting subject 19). On examination, the diets of the above subjects were considered to be adequate but certainly not excellent. About three-quarters of the bread eaten was white. Although there was no doubt increased loss of vitamin B_1 during the large-quantity cooking, the intake of the college subjects was approximately equal to that of the home subjects because they ate more daily and their menu included more meat and vegetable dishes and less breadstuffs.

The average excretion of those subjects residing in lodgings was 90 μg., a figure significantly low. The range in this group was 43–158 μg., compared with a range of 103–441 μg. obtained from the college and home subjects. Chiefly for economic reasons the students were obliged to dwell in these cheaper lodgings rather than in the relatively expensive university colleges. Consequently the diet supplied was of a poorer standard both in quality and quantity, non-protective foods taking the place of more costly protective foods. This was reflected in the low vitamin B_1 excretion values consistently obtained from these subjects.

The subjects examined in the present study may be classified as sedentary to moderately active. The optimal daily allowance recommended by the Food and Nutrition Board, National Research Council, U. S. A. ('41), for a 70-kg. man in this group is 1.5–1.8 mg. Work done since the Council's recommendations were announced has given a more specific basis for the setting of the minimal requirement. Melnick ('42), from urinary excretion levels obtained from subjects on controlled vitamin B_1 intake, concluded that 350 µg. per 1000 calories should be regarded as the minimum daily requirement. Allowing a 50% margin for safety, the recommended allowance becomes approximately 500 µg. per 1000 calories. Williams and Mason ('41, '42), from their data consider that 400 µg. per 1000 calories is the minimal requirement and that the optimal intake of vitamin B_1 is not more than 1 mg. per 1000 calories.

The calorific intake of the subjects was approximately 3000 calories daily. According to the above standards, 1.05 to 1.2 mg. of vitamin B_1 would be required to satisfy their minimal requirement. Allowing the 50% margin for safety, 1.6–1.8 mg. would be necessary to attain their optimum intake similar to the figure stated above by the Food and Nutrition Board.

On consideration of the results and conclusions put forward by various workers (compare table 5), on the relation of the urinary excretion of vitamin B_1 to the dietary supply, it appears to be generally agreed that a diet supplying approximately 1 mg. is associated with an excretion of above 100 µg. Excretion below this level indicates deficiency. Jolliffe et al. ('39) in their study of induced vitamin B_1 deficiency, found that subjective symptoms did not occur unless the excretion fell below 100 µg.

The urinary excretions of the home and college subjects were all above 100 µg., showing that these individuals were receiving a sufficient supply of the vitamin to satisfy their minimal needs; in many cases the urinary value indicated that probably more than 1 mg. was supplied by the diet. In

these cases the substitution of the white bread by 100% whole meal would raise the intake of vitamin B_1 considerably nearer to the optimal level. Obviously the lodgings' diets did not supply enough vitamin B_1 for even minimal needs, the average excretion being only 90 μg. For these subjects who could not afford to seek better residences and who could do little to improve the general standard of the diet supplied, it was recommended that part or all of the jam in their diet be substituted by peanut butter, and 1–2 tablespoons of wheat germ be eaten daily, an addition of approximately 350 μg. of vitamin B_1.

The basal 24-hour sample, according to Mason and Williams ('42) and Melnick and Field ('42), is as reliable and gives as much information as the test dose procedure. As the latter was not essential, few determinations were made, owing to the inconvenience entailed in the collection of 24-hour urine samples. Table 4 gives the results from five subjects before and after an oral test dose of 1 mg. Those subjects living in lodgings retained more of the test dose, showing that their reserve stores were depleted, as was indicated by the low basal 24-hour excretion values. Nine to thirteen per cent of the dose was excreted by the college and home subjects, a response within the normal range for a test dose of this magnitude (compare Borson, '40).

SUMMARY

The vitamin B_1 contents of some New Zealand cereal products were estimated; an average whole wheat gave a value of 4.3 μg., and an average white flour, 2.0 μg. per gram. Bread prepared from 80% extraction flour had a vitamin B_1 content almost twice as great as that made from the 70% extraction product. Of the twenty-five breakfast cereals examined, the oat and wheat porridge products proved to be good sources of vitamin B_1. The "ready-to-eat" cereals, during processing, had lost most, or all, of the vitamin contained in the original grain. The advertising claims made in these cases were erroneous and misleading.

The 24-hour vitamin B_1 urinary excretion values of thirty-nine normal subjects were determined. From the application of suggested standards relating excretion to intake, their level of vitamin B_1 nutrition has been assessed. The excretions for the home and college subjects ranged from 103–441 μg.; their dietary supply was considered to be adequate. A range of 43–158 μg., average value of only 90 μg., was found for those subjects residing in cheap lodgings. The diet in the majority of these cases did not supply sufficient vitamin B_1 for even minimal needs.

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THE CAROTENE CONTENT OF TOMATOES AS INFLUENCED BY VARIOUS FACTORS[1]

G. H. ELLIS AND K. C. HAMNER
United States Plant, Soil and Nutrition Laboratory, Ithaca, New York

ONE FIGURE

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INTRODUCTION

It is evident from a review of the literature (Hamner and Maynard, '43) that values for the carotene content of tomatoes show considerable variation. Some of these variations are doubtless due to the methods of analysis used. Several investigators working independently with differing methods reached conclusions indicating that the carotene content of tomatoes is influenced by different factors such as degree of ripeness, variety, and perhaps environmental factors. In the investigations reported here, an attempt has been made to determine the relative importance of various factors governing carotene content.

EXPERIMENTAL

The assay method used which is described in detail elsewhere (Ellis et al., unpublished) was essentially as follows: A separation of diacetone and acetone soluble pigments, after transferring to petroleum ether, is made on a Tswett column consisting of a mixture of magnesium oxide and diatomaceous earth. The top band of pigments (largely lycopene) is discarded, the lower band (largely β carotene) is eluted with petroleum ether containing 4% ethanol and the pigment concentration determined in a photoelectric colorimeter previ-

[1] The authors gratefully acknowledge the guidance and suggestions of Dr. L. A. Maynard.

ously calibrated with β carotene. Thus the method is somewhat empirical but its use determines a group of carotenoid pigments consisting mostly of β carotene. It gave good agreement with a rat bio-assay performed on a sample of canned tomato purée. An important feature of the method is its rapidity, which enabled us to complete a great many analyses within a relatively short period of time. It was also possible to complete the initial stages of the extraction and transfer the pigments to petroleum ether within an hour after the fruit was sampled.

In most cases analyses were made upon each individual tomato sampled. In certain experiments, the provitamin A extracts of the individual tomato fruits (in petroleum ether) were made to a definite volume and aliquots equivalent to a given weight of fresh material were taken. Several of these were combined to give a composite extract representative of several tomatoes. In these instances, the presentation of data includes figures for the number of tomatoes sampled as well as the number of carotene analyses completed.

Sampling technique and distribution of carotene in the tomato

Since many of the tomatoes upon which carotene analyses were made were also analyzed for other constituents, it was impossible to use the entire fruit for each analysis. Therefore, it was proposed that an estimate of the carotene content be obtained from the analysis of a transverse section taken from the equatorial region. In order to determine whether or not such a sample would be representative, the following data were obtained.

Eighteen ripe tomatoes of nearly uniform size were each cut into three portions, namely, (1) a transverse section of about 20 gm. at the equatorial region, (2) the remaining blossom end and (3) the remaining stem end. The average results of carotene analyses, eighteen in each case, and expressed as micrograms per gram fresh weight, were as follows: blossom end, $3.86 \pm .155$; median portion, $4.29 \pm .194$; and stem end, $4.93 \pm .167$; for the entire fruit, $4.49 \pm .150$.

These data show a greater concentration of carotene in the stem end than in the blossom end of the fruit, with the transverse segment giving intermediate values. The difference between the carotene concentration of the median section and the entire fruit is not statistically significant, partly because of the variability among the individual tomatoes. The standard deviation for an individual determination on a median portion as compared with the whole fruit is $\pm 6.8\%$. The mean obtained by analysis of the median portions of eighteen fruits is 95.6% of that obtained by analysis of the same entire fruit.

Effect of fruit size

Differences of considerable magnitude have been noted in carotene content when various tomatoes are compared, even though the fruit were picked from the same vine at what appeared to be comparable stages of ripeness. No explanation is as yet available for these variations. That they are not correlated with differences in fruit size is demonstrated by the following data taken from analyses made in connection with another study. All tomatoes were picked on the day when the entire surface was red. All were from plants of an inbred strain of Bonny Best grown in sand cultures. The average results of carotene analyses of fruit of various sizes expressed as micrograms per gram fresh weight were as follows: 10 to 40 gm., $4.55 \pm .138$; 41 to 70 gm., $4.58 \pm .093$; 71 to 100 gm., $4.99 \pm .135$; 101 to 130 gm., $4.94 \pm .123$. The number of analyses made in obtaining these averages was 109, 134, 93 and 52, respectively. While these data indicate that small fruit may contain slightly less carotene per gram fresh weight, the differences are not great and probably have little practical significance.

Effect of the supply of macronutrient[2] *elements*

In a previous publication (Hamner et al., '42) descriptions have been given of experiments designed to determine the

[2] The term macronutrients as used in this paper refers to the six nutrients (Ca, K, Mg, NO_3, SO_4, and PO_4) in contrast to the micronutrients (Cu, Mn, B, Zn, etc.). The distinction between these two groups of nutrients is based upon the fact that the former group is needed by the plants in much greater amounts than the latter group.

effects of mineral nutrition upon the ascorbic acid content of tomatoes. The fruit which were analyzed for that report were also analyzed for carotene. The results of some of these analyses are reported now. Since the experimental details already have been reported, they are not included here.

In experiment I, Bonny Best tomatoes were grown in sand cultures in the greenhouse during the fall and early winter. In experiment II plants of an inbred strain of Bonny Best were grown in sand cultures out-of-doors during the summer. In both experiments, the design was such that the effects of variations in the relative proportions of the macronutrient elements supplied to the plants could be measured. The results of the carotene analyses of experiment I are not reported in detail here since the data were very similar to those obtained in experiment II described later. The average of over 200 carotene analyses obtained in experiment I was $3.59 \pm .099$ µg. per gram fresh weight. No correlation was found between treatment and carotene content.

In experiment II, 1044 tomato plants were grown in sand culture with eighty-seven different nutrient solutions. The compositions of the nutrient solutions varied with respect to the relative proportions of the macronutrients: NO_3, PO_4, SO_4, K, Ca, and Mg. In all cases where sufficient fruit were produced the first two fruit to ripen on each plant were sampled and the extracts of fruit from three plants combined to give two analyses, one for the first and one for the second fruit. The results of the carotene analyses are given in figure 1. In the legend is given a brief discussion of the nutrient treatments. These results were subjected to a statistical analysis. The F values obtained from the observed means of replicates in an analysis of variance were significant in both triangles when odds of 99:1 were accepted. From an inspection of the data in the cation triangle, it is evident that at least part of this significance was contributed by the high mean (8.5 ± 2.60) in the treatment near the potassium apex and by the low mean ($3.8 \pm .43$) in the treatment immediately adjacent. The number of analyses in each of these treatments

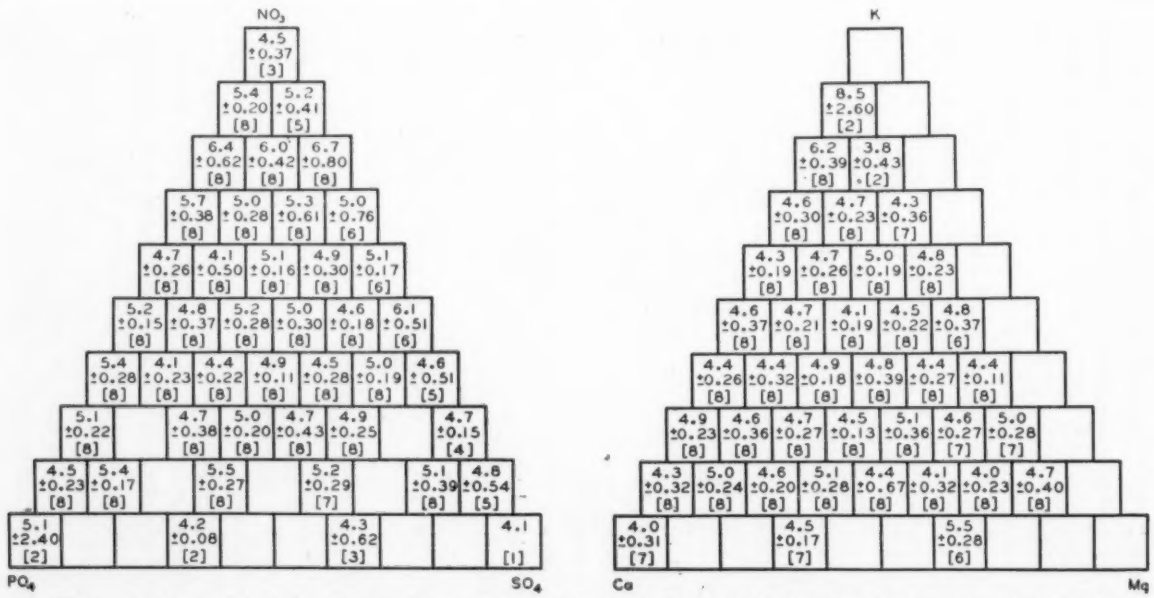

Fig. 1 Carotene content in micrograms per gram fresh weight of tomato fruit produced in experiment II. Treatment means are given together with their standard errors. The number of analyses in each treatment is recorded in brackets. Each of the squares in each triangle represents a nutrient treatment in sand culture supplied to twelve plants. In the anion triangle on the left, the relative proportions of the anions are varied while the cation supply is constant. The relative amount of a particular anion is indicated by the proximity of the given treatment to the apex where that anion is listed. Thus, each apex represents a treatment supplied with the single anion listed at that apex, and each base represents a series of treatments deficient in the anion at the opposite apex. In the cation triangle at the right, the anions were constant, and the relative proportions of the cations varied in a manner similar to the anion relations discussed above. For details of the experimental work, see Hamner, Lyon and Hamner ('42).

were only two and do not permit any sound conclusions relative to the effects of these treatments. There are no evident trends in the cation triangle which could be correlated with nutrient composition.

In the anion triangle, it seems likely that the significance was related to the high means obtained in treatments where relatively large amounts of nitrates were supplied to the plants. The conclusion seems justified that variations in the supply of nitrogen to tomato plants result in variations in the carotene content of fruit. The magnitude of the variations in carotene related to nitrogen supply, however, is not great and probably has little practical significance. Thus, wide variations in the supply of the macronutrient elements to the plants produced very little effect upon the carotene content of the fruit although these treatments did markedly affect the growth and fruitfulness of the plants (Hamner et al., '42) and the mineral content of the fruit (Lyon et al., '42). The average value for over 500 analyses of experiment II was $4.88 \pm .072$ µg. per gram.

The most significant differences obtained were between the average values of the two experiments. The carotene content of fruit produced in the greenhouse during the winter averaged 26.4% lower than that of tomatoes obtained out-of-doors during the summer. These differences are discussed later.

Effect of location where grown

Carotene analyses were also made on fruit produced in experiments III and IV described in the publication (Hamner et al., '42) mentioned above. Tomato plants of the same inbred strain of Bonny Best were grown in sand culture and in soil at five widely separated locations in the United States. At all locations, the plants grown in sand culture were watered with nutrient solutions of similar composition and at each location, the plants in soil were grown in good garden soil. The results of the analyses are given in table 1. In the same experiment, tomato plants were grown at Ithaca, New York,

in large samples of soil obtained from three of the above locations. The results obtained for the fruit from these plants are given in table 2. The data in table 1 indicate that the location where the plants are grown possibly has an effect

TABLE 1

Carotene content of tomato fruits from plants grown in soils or in sand culture under different environments.[1]

LOCATION	PLANTS GROWN					
	In naturally occurring soils			In sand cultures		
	γ/gm. Fresh weight Mean S.E.	No. of analyses	No. of fruit	γ/gm. Fresh weight Mean S.E.	No. of analyses	No. of fruit
Ithaca, New York	4.54 ± .467	5	17	4.88 ± .072 [2]	83	
Riverside, Calif.	5.27 ± .501	4	23	4.53 ± .267	4	17
Berkeley, Calif.[3]	4.05 ± .142	8	8	4.17 ± .293	10	10
Lake Geneva, Wisc.[4]	4.99 ± .392	10	10	4.57 ± .493	11	11
Cheyenne, Wyoming	4.28 ± .091	11	11			

[1] To avoid losses of carotene during shipment, extracts of the fruit were made at each location by one of us, and the extracts sent to Ithaca for analysis.

[2] This figure represents the average value of the treatments in experiment II (fig. 1).

[3] Plants grown in greenhouse.

[4] Plants grown in undisturbed soil.

TABLE 2

Carotene content of fruits on plants grown in different soils but in the same climatic environment at Ithaca.

SOIL FROM	γ/gm. Fresh weight Mean S.E.	NO. OF FRUIT	NO. OF ANALYSES
Ithaca, New York	4.54 ± .467	19	5
Berkeley, California	5.06 ± .349	21	5
Cheyenne, Wyoming	4.63 ± .177	26	5
Lake Geneva, Wisconsin	4.56 ± .366	22	5

upon the carotene content of the fruit. It should be noted, however, that the lowest values obtained were at Berkeley, California, and at this location, the plants were grown in the greenhouse. The possible significance of this is discussed later. Significant differences in carotene content were not

obtained when the fruit produced by plants in sand culture were compared with those in soil. This would indicate that the possible differences owing to location were probably correlated with climatic environmental conditions rather than with differences in mineral nutrient supply. This conclusion is further substantiated by the fact that plants grown at Ithaca, New York, in four different soils (table 2) produced fruit with almost identical average carotene content.

During the summer of 1942, additional data were obtained relative to the effect of location on carotene content. Fruit of the Marglobe variety were picked from plants grown in soil at several locations when nearly red-ripe. They were

TABLE 3

Carotene content of Marglobe tomato fruit grown in soil at several locations and shipped to Ithaca for analysis.

LOCATION WHERE GROWN	NO. OF FRUIT SAMPLED	NO. OF ANALYSES	CAROTENE IN γG./GM. FRESH WEIGHT
Madison, Wisconsin	14	2	3.9
St. Paul, Minnesota	10	2	4.2
Holgate, Ohio	13	2	4.6
Lansing, Michigan	15	2	4.9

packed carefully in paper and sent at once by express to Ithaca. Upon arrival, all injured fruit were discarded and the remainder analyzed for carotene. If, upon arrival, some of the fruit were slightly green, these were permitted to stand at room temperature until red over the entire surface. In no case did this require more than 2 days. The results of the analyses are given in table 3. These data give little further evidence that the location where the plants are grown influences carotene content.

Effect of variety and fertilizer treatment

During the summer of 1942, analyses were made on fruit of three varieties of tomatoes grown at the New York State Experiment Station, Geneva, New York. The details of the experimental plan, fertilizer treatments, growth and yield

data and related matters, will be reported by Dr. C. B. Sayre in a separate publication. The design of the experiment was that of blocks selected at random and covered seven fertilizer treatments and three varieties. There were three replicates of each treatment for each variety. Seven fruits were combined from each replicate to give a single analysis. The data were reduced by means of the analysis of variance. The results of these calculations are given in table 4. The mean values for carotene content of each treatment are given in

TABLE 4

Results of analyses of variance for fertilizer experiment at Geneva, New York.

VARIATION DUE TO	DEGREES OF FREEDOM	SUM OF SQUARES	VARIANCE	"F" VALUE	"F" VALUE REQUIRED FOR "P" VALUE OF 0.01
Total	61				
Treatment	6	9.2598	1.5433	1.64	3.67
Blocks	2	0.8289	0.4145	0.44	5.61
Varieties	2	15.5563	7.7782	8.28	5.61
Treatment × variety	12	9.7303	0.8109	0.86	3.03
Variety × block	4	4.5985	1.1496	1.22	4.22
Treatment × block	12	14.3424	1.1952	1.27	3.03
Treatment × variety × block (error)	23	21.5956	0.9389		

table 5. It is apparent that while fertilizer treatment has no evident effect on carotene, the variety of tomato can result in small differences. Thus, tomatoes of the Stokesdale variety are significantly richer in carotene than New York State, with the results for Rutgers intermediate between the two.

Effect of degree of ripeness

Most investigators (Hamner and Maynard, '43) are in agreement that the carotene content of tomatoes increases during ripening and that fruit ripened on the vine are higher in carotene than fruit picked while green and allowed to ripen in storage. It was thought desirable to check these findings with the particular method of analysis which we have been using.

Fruits of the Pritchard variety were supplied by the Department of Vegetable Crops at Cornell University from plants grown in the departmental gardens.[3] All fruits were picked on September 2, 1942. Fruit which showed neither injury nor broken skin and which were soft and seemed obviously overripe are so called (see table 6). Sound firm fruit which were red over the entire surface are called red-ripe fruit. Those partially red-colored are called pink. Those

TABLE 5

Carotene content of fruit produced on plants grown at Geneva, New York, on soil receiving seven different fertilizer treatments.

PLOT	FERTILIZER APPLICATIONS [1]	VARIETY (CAROTENE IN μG./GM. FRESH WEIGHT)			
		Stokesdale	Rutgers	Nystate	Treatment mean [2]
H	No fertilizer	5.4	4.0	3.5	4.3
A	0–20–10 at 600 lb. per acre	6.5	4.6	4.2	5.1
B	5–20–10 at 600 lb. per acre	4.2	4.0	4.2	4.1
D	20–20–10 at 600 lb. per acre	5.3	4.0	4.0	4.4
E	5–20–10 at 600 lb. per acre + 60 lb. of MgO as $MgSO_4$	4.9	4.7	3.5	4.4
I	5–20–20 at 600 lb. per acre	4.6	5.2	4.1	4.7
J	3–12–6 Premium Grade at 1000 lb. per acre	5.3	5.6	4.5	5.1
	Varietal mean [3]	5.2	4.6	4.0	

[1] Figures show percentages of available N, P, and K as total N, P_2O_5, and K_2O obtained by analysis of fertilizer.

[2] The generalized error taken from the analysis of variance for any inter-treatment comparison is 0.54.

[3] The generalized error taken from the analysis of variance for any inter-varietal comparison is 0.37.

of an even green color and about one-half the size of the average ripe fruit are called immature green. A number of these fruit at the various stages of ripeness were analyzed immediately for carotene. About 400 fruit were selected at a stage which might be termed mature green. These fruit were of a large size and a light-green color with areas of the surface which were almost white. None of them showed red

[3] Thanks to the courtesy of Dr. H. C. Thompson.

coloration. Twenty of these mature green fruit were selected for immediate analysis and the remainder were divided into five lots. A lot of fifty was placed at each of the following storage temperatures: 65°, 70°, 80° and 90°F. An additional lot of 150 fruit were placed in storage at 75°. At each of the storage temperatures, the fruit were analyzed as soon as

TABLE 6

Carotene content of fruit at different stages of ripeness and after storage at different temperatures.

TREATMENT	STAGES OF RIPENESS	NUMBER OF ANALYSES	NUMBER OF FRUIT	CAROTENE μG./GM. FRESH WEIGHT
Initial harvest	Immature green	3	20	1.7 ± .05
	Mature green	3	20	1.8 ± .29
	Pink	3	20	4.0 ± .55
	Red-ripe	3	20	4.0 ± .60
	Overripe	1	8	3.7
Stored at 65°F.	Red-ripe	6	33	2.1 ± .32
Stored at 70°F.	Red-ripe	7	38	1.7 ± .25
Stored at 75°F.	Red-ripe	14	119	2.2 ± .16
Stored at 80°F.	Red-ripe	8	43	2.7 ± .37
Stored at 90°F.	Ripe [1]	7	37	1.9 ± .37
Stored at 75°F.	Mature green	2	10	1.4
and removed after	Pink	2	8	1.5
10 days storage	Red-ripe	2	17	2.4
Stored at 75°, 1 to 8 days	Red-ripe	2	11	3.0
Stored at 75°, 9 to 12 days	Red-ripe	5	35	2.3 ± .24
Stored at 75°, 13 to 19 days	Red-ripe	7	73	1.9 ± .23

[1] Did not become red-ripe. Analyzed when yellowish pink.

they turned red over the entire surface. At the higher temperatures, some of the fruit did not turn red but rather changed from a green to a yellowish pink. These were analyzed when they had lost most of their green color. Ten fruit stored at 75° for 10 days were analyzed at the pink stage and an additional ten while still green.

The results of the analysis are given in table 6. These data indicate that vine-ripened fruit are much higher in carotene than fruit picked green and ripened in storage, regardless of the storage temperature. In fact, fruit picked green and allowed to ripen in storage increased only slightly in carotene. There are indications that the fruit which ripened first during storage are higher in carotene than those which ripened later.

Miscellaneous observations

Smith ('36) reported that tomatoes produced in the greenhouse were lower in carotene than those produced outside. This conclusion is substantiated by numerous observations of our own. Plants of the inbred strain of Bonny Best grown in soil outside gave fruit which averaged 4.7 ± .07 μg. per gram (twenty analyses), while comparable plants grown at the same time in similar soil in the greenhouse gave an average of 3.8 ± .52 μg. per gram (six analyses). Plants of the inbred strain of Bonny Best grown in the greenhouse during the winter months (November to January) produced fruit with an average carotene content of 3.8 ± .33 μg. per gram based on sixteen fruit and four analyses. In the experiments summarized in table 1, it was found that the lowest value obtained was 4.0 ± .14 μg. per gram for the fruit produced at Berkeley, California. These fruit were produced in the greenhouse during the summer. Fruit produced in the greenhouse in the winter in experiment I averaged 3.6 ± .10 μg. per gram. Thus, it would appear that fruit produced in the greenhouse are relatively low in carotene whether they are grown during the winter or summer.

An experiment was conducted to determine the effects of certain variations in light quality upon carotene content. Tomato plants of the inbred strain of Bonny Best were grown from seed to maturity in rooms where they received artificial illumination from fluorescent type lamps. Eight plants received light from "daylight fluorescent" tubes and eight from "white fluorescent" tubes. Thus, one set of plants received

relatively more blue light than did the other.[4] The intensities of illumination of each set of plants were the same when measured by a Weston foot-candle meter, and were of approximately 1200 foot-candles (the intensities near the top of the plants were somewhat higher than at the base). The amount of growth and fruit production was approximately equal in both sets of plants. The carotene content of the fruit produced by the two sets was very nearly the same, being 4.57 ± .191 μg. (sixteen analyses) under the "daylight" tubes and 4.33 ± .172 μg. per gram (fourteen analyses) under the "white tubes."

In view of the fact that fruit picked green and permitted to ripen in storage as well as fruit produced in the greenhouse, had low carotene values, it was of interest to analyze fruit from the market during the winter. On December 9, 1942, two lots of ripe fruit were obtained from the local market at Ithaca, New York. One lot was produced in the south and probably from vines grown outside. The other lot was from vines grown in hot-houses in Ohio. The carotene contents of these two lots were: southern-grown 2.4 ± .16 μg. per gram for seventeen fruit, and hot-house grown 1.9 ± .16 μg. per gram for fourteen fruit. The fruit grown in the hot-house were slightly lower in carotene than those grown in the south, but both values are about half that which might be expected for vine-ripened fruit.

DISCUSSION

In much of the work discussed above, a single variety of tomatoes was used (Bonny Best) and most were of an inbred strain. Ripe fruit from this variety were produced on plants grown at different locations, in different soils, in many different nutrient solutions, under artificial illumination, in summer (outside and in the greenhouse), and during the winter (in the greenhouse). The lowest values for carotene content were obtained for fruit produced in the greenhouse whether grown during the summer or winter. None of the

[4] General Electric Co., Mazda Lamps, Bulletin LD-1, September, 1940.

other variations in environment seemed to affect carotene content very much when the fruits were allowed to ripen on the plant.

Results in table 5 indicate that variety influences carotene content. However, these differences are not great. All varieties observed were large-fruited, commercial forms of the same color type. If other varieties had been included in the above study, it may be that greater varietal differences would have been found. In this report, data on the carotene content of six varieties are included. While the values of these different varieties are not strictly comparable, it may be noted that, regardless of variety, vine-ripened tomatoes produced outside during the summer are in fair agreement. By far, the lowest values obtained were on those fruit (1) picked green and ripened in storage (table 6), and (2) obtained on the market in the winter. Presumably, the latter were also picked green, and some were from the greenhouse.

SUMMARY

1. Several thousand tomato fruit were analyzed for carotene. Fruit were included from plants grown in soil and in sand cultures at various locations and under differing environmental conditions. Attempts were made to correlate the carotene content of the fruit with factors such as fruit size, degree of ripeness, mineral nutrient supply of the plant, soil conditions, and variety.

2. Analyses of ripe tomato fruit show a greater concentration of carotene in the stem-end than in the blossom-end.

3. Of several hundred ripe fruit analyzed for carotene, there was little correlation between fruit size and carotene content. Large fruit were only slightly richer in percentage carotene than small fruit.

4. Wide variations in the supply of the macronutrient elements to tomato plants growing in sand cultures produced only slight variations in the carotene content of the fruit even though the variations in nutrient supply greatly affected growth and fruitfulness of the plants. There were indications

that increasing supplies of nitrates to the plants resulted in fruit of increasing carotene content, but the magnitude of the variations in carotene was not great.

5. Differences in carotene content were correlated with varietal differences, but the particular varieties reported here did not differ greatly.

6. Ripe fruit produced in the greenhouse, whether in summer or winter, were lower in carotene than fruit produced outside during the summer.

7. Fruit which were picked green and ripened in storage were very much lower in carotene than vine-ripened fruit. From a practical standpoint, this difference seems to be the most important one noted in this report.

8. Ripe fruit obtained from a local market during the winter contained about half as much carotene as might be expected in vine-ripened fruit in the summer.

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THE ROLE OF "FOLIC ACID" AND BIOTIN IN THE NUTRITION OF THE RAT

A. D. WELCH AND LEMUEL D. WRIGHT
WITH THE TECHNICAL ASSISTANCE OF K. L. SPRAGUE
Nutritional Laboratories, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pennsylvania

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The recognition of the importance of thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, inositol, p-aminobenzoic acid and choline as essential components of the diet has made it possible to rear rats on highly purified rations at a growth rate approximating that obtained on good commercial foods (Jukes, '40; Unna et al., '41; Richardson et al., '41; Martin, '42). Evidence is now accumulating, however, that purified rations containing these added factors are adequate, not because they include all exogenous substances required for growth, but because the synthetic activity of certain intestinal bacteria also supplies essential factors. Such factors are normally provided by a diet composed of relatively crude components.

When certain sulfonamides are incorporated into highly purified diets, typical signs of dietary deficiency are produced (Black et al., '41; Mackenzie et al., '41; Daft, Ashburn and Sebrell, '42; Daft, Ashburn, Spicer and Sebrell, '42; Welch, '42). For this purpose sulfonamides which are poorly absorbed from the intestinal tract have proved particularly useful. Black, McKibbin and Elvehjem ('41) employed sulfaguanidine (sulfanilylguanidine) at levels of from 0.5 to 2.0% in a purified diet fed to rats and noted that the growth rate of the animals was markedly reduced. The addi-

tion of p-aminobenzoic acid to the diet was only partially effective in preventing the decrease in rate of growth caused by the sulfonamide. On the other hand, these workers found a liver extract to be completely effective in counteracting the effect of the drug. Another sulfonamide, succinylsulfathiazole (2-(N^4-succinyl-sulfanilamido)-thiazole), which is less toxic than sulfaguanidine (Welch et al., '42) and which is absorbed to an even lesser degree from the alimentary tract was found by Welch ('42) to produce a similar effect on the growth of the rat when added to a highly purified ration to the extent of 1.0 to 2.0%. Although the addition of liver extract to a diet containing succinylsulfathiazole was effective in maintaining a normal growth rate, p-aminobenzoic acid was reported as failing to exert its anticipated influence on the action of the sulfonamide.

The extremely low toxicity of succinylsulfathiazole, the very poor absorption of the drug from the alimentary tract, and the pronounced effect of the compound on certain intestinal bacteria, suggested that this drug might be useful in the detection of those factors normally synthesized by the bacteria of the intestinal tract. The work herein reported describes the identification of two factors which must be supplied to the rat to permit good growth and development when succinylsulfathiazole is incorporated in a synthetic ration adequate in the well-recognized dietary essentials for this species. During the progress of this work Black et al. ('42) and Nielsen and Elvehjem ('42) reported that the growth inhibition produced by succinylsulfathiazole can be largely overcome by dietary supplements of "folic acid" concentrate [1] and biotin. Under somewhat different conditions additional evidence has been accumulated in this laboratory which points to the essentiality of "folic acid" and biotin as dietary constituents when succinylsulfathiazole is incorporated in highly purified diets.

[1] The term "folic acid" refers to concentrates which furnish an essential growth factor for *L. casei*. Although the activity of the concentrates in rats is attributed to their "folic acid" content, other substances are present, since the materials used contained only a small proportion of actual "folic acid."

EXPERIMENTAL

Experiments directed toward the identification of the factors which are required by the rat, apparently synthesized by intestinal bacteria, and present in liver extract, have involved the testing of many substances of known structure and the assay of fractions derived from various crude sources of active material.

During the progress of the study the basal diets have been modified from time to time as indicated in table 1. Rations

TABLE 1

Composition of basal diets employed in feeding experiments.

	S–1	S–4–A	S–4–B	S–7
	gm.	*gm.*	*gm.*	*gm.*
Casein (SMA or Labco)	18.0	18.0	18.0	18.0
Hydrogenated cottonseed oil (Primex)	...	...	...	10.0
Corn oil	2.0	2.0	2.0	2.0
Sucrose	76.0	76.0	76.0	59.9
Salts[1]	4.0	4.0	4.0	4.0
Cellu flour	...	...	...	4.0
A, D and E concentrate[2]	*	*	*	0.08
Choline chloride	0.2	0.1	0.1	0.1
	mg.	*mg.*	*mg.*	*mg.*
Thiamine hydrochloride	3.0	3.0	30.0	0.2
Riboflavin	3.0	3.0	30.0	0.4
Pyridoxine hydrochloride	3.0	3.0	30.0	0.2
Nicotinic acid	2.5	2.5	25.0	4.0
Calcium pantothenate	2.0	2.0	20.0	4.4
p-Aminobenzoic acid	...	...	...	4.0
Inositol	...	...	...	8.0

[1] Osborne and Mendel ('13) or Hubbell, Mendel and Wakeman ('37).

[2] Compounded as follows: fish liver concentrate containing 450,000 U.S.P. units of vitamin A and 90,000 U.S.P. units of vitamin D per milliliter, 7 gm.; α-tocopherol, 2 gm.; corn oil, 41 gm.

* Animals in these groups were given 2 drops per week of percomorph oil fortified with α-tocopherol (1 mg. per 25 mg. oil).

containing succinylsulfathiazole were prepared by substituting it for an equivalent amount of sucrose. The animals used in the growth studies were male black and piebald weanling rats.[2] Each animal was caged individually over wide-mesh screening. Food and water were supplied ad libitum.

[2] Sunny Hill strain.

Succinylsulfathiazole, when incorporated to the extent of 5 or 10% in a complete diet of natural materials,[3] caused no demonstrable effect on the rate of growth of rats, as compared with the growth rate of animals maintained on the same diets without drug (table 2). The animals appeared normal in every way and no evidence of pathological changes was demonstrable histologically. However, much smaller amounts of succinylsulfathiazole (1 or 2%) were effective in producing marked inhibition of growth when added to purified rations which were otherwise adequate in their content of the hitherto recognized essential dietary factors. The drug appeared to have little if any effect on the growth rate during the first or second week of feeding; evidence of the

TABLE 2

Effect of succinylsulfathiazole, when added to a complete commercial diet,[1] on the growth rate of rats.

LEVEL OF ADDED SUCCINYL-SULFATHIAZOLE	NUMBER OF ANIMALS	ORIGINAL WEIGHT[2]	DURATION OF EXPERIMENT	GAIN IN WEIGHT	
				Total[2]	Per week
		gm.	*days*	*gm.*	*gm.*
None	10	113 ± 4.1	33	98 ± 6.4	14.9
5%	10	115 ± 4.4	33	106 ± 3.8	16.1
None	7	75 ± 8.8	44	152 ± 10.6	24.2
10%	8	77 ± 4.5	44	167 ± 6.7	26.5

[1] Wayne brand.

[2] These weights are expressed as averages, together with the standard error: $\sqrt{\frac{\Sigma\ d^2}{n\ (n-1)}}$

inhibition of growth was apparent during the third and fourth week and was marked thereafter (table 3). Other effects on the animals were seen frequently but irregularly. These included alopecia, usually restricted to a definite pattern, spectacled eyes and pan-ophthalmitis, porphyrin-caked whiskers, and achromotrichia.

It was reported (Black et al., '41) that p-aminobenzoic acid (PABA) is partially effective in combatting the growth-inhibiting effect of sulfaguanidine. When succinylsulfathiazole, at a level of 1%, was employed as the inhibitor of growth, the administration of PABA also appeared to have only a partial effect in suppressing the action of the sulfonamide

[3] Either Wayne brand or Purina brand.

(table 4). Methionine, which is known to have some antisulfonamide action (Kohn and Harris, '41), was ineffective in promoting growth when added to the basal diet (succinylsulfathiazole, 1%) to the extent of 0.6%; inositol was without effect when fed at a level of 0.25%. Biotin, at a level of 20 μg. per cent, appeared to have a slight stimulating effect on growth. A yeast concentrate and a liver extract, each at a level of 1%, partially antagonized the effect of the sulfonamide, while an extract of rice polishings, at the same level, was possibly more active. The effect of proteose-peptone [4]

TABLE 3

Effect of succinylsulfathiazole, when added to a synthetic ration,[1] on the growth rate of rats during a period of 6 weeks.

LEVEL OF ADDED SUCCINYL-SULFATHIAZOLE	NUMBER OF ANIMALS	AVERAGE WEEKLY GAIN IN WEIGHT DURING			TOTAL GAIN IN WEIGHT [2]
		0–2 wk.	2–4 wk.	4–6 wk.	
%		gm.	gm.	gm.	gm.
0	20(0) [3]	23.0	15.0	11.7	99.5 ± 4.3
1	19(1)	22.4	11.4	3.9	75.5 ± 2.8
2	10(0)	21.8	5.5	0	53.3 ± 2.1
5	7(3)	17.9	7.7	−3.6	44.1 ± 4.5

[1] Diet S–1 was employed.

[2] These weights are expressed as averages, together with the standard error.

[3] Figures in parentheses indicate the number of additional animals which failed to survive the 6-week test period; data on these animals are not included in the growth summary.

(5%) was very striking; not only was the action of the drug abolished, but the gain produced during a 43-day period clearly exceeded that noted for the basal diet without the drug. That this effect was not due to the additional protein derivatives made available to the animals was shown by the absence of the effect when an equivalent amount of casein was fed.

Before initiating fractionation procedures to characterize the new factor or factors, there were tested several compounds which have been implicated in one way or another as possible essential dietary constituents or growth factors for various species of animals or bacteria. The following compounds or substances were found to be inactive in promoting growth,

[4] Difco brand.

TABLE 4

Effect of various supplements on growth of rats fed a purified diet containing succinylsulfathiazole.

SUPPLEMENT	LEVEL OF SUCCINYL-SULFA-THIAZOLE	DIET USED	NUMBER OF ANIMALS	DURATION OF EXPERI-MENT	GAIN IN WEIGHT	
					Total [1]	Per week
	%			*days*	*gm.*	*gm.*
None	0	S–1	20	43	99.5 ± 4.3	16.2
None	1	S–1	19	43	75.5 ± 2.8	12.3
PABA (0.2%)	1	S–1	9	43	87.6 ± 3.1	14.3
dl-Methionine (0.6%)	1	S–1	8	37	61.4 ± 2.7	11.6
Inositol (0.25%)	1	S–1	10	43	77.6 ± 3.6	12.6
Biotin concentrate (20 μg. %)	1	S–1	9	43	82.6 ± 1.8	13.4
Yeast concentrate (1%)	1	S–1	10	43	85.9 ± 7.4	14.0
Rice polish concentrate (1%)	1	S–1	9	34	81.2 ± 4.7	16.7
Liver extract (1%)	1	S–1	9	43	87.7 ± 2.6	14.3
Proteose peptone (5%)	1	S–1	10	43	122.3 ± 6.9	19.9
None	0	S–4A	9	42	103.0	17.2
None	2	S–4A	7	42	46.1	7.7
Proteose peptone (1%)	0	S–4A	4	42	118.5	19.7
Proteose peptone (5%)	0	S–4A	5	42	144.0	24.0
Casein (5%)	0	S–4A	4	42	105.3	17.6
Proteose peptone (1%)	2	S–4A	5	42	65.0	10.8
Proteose peptone (5%)	5	S–4A	5	42	76.0	12.7
Calcium pantothenate (18 mg. %)	0	S–4A	5	42	101.6	16.9
Calcium pantothenate (18 mg. %)	2	S–4A	3	42	33.3	5.6
None	0	S–4B	9	42	92.7	15.4
None	2	S–4B	7	42	30.6	5.1
Inositol (0.25%) + PABA (0.25%)	0	S–4B	5	42	101.2	16.9
Inositol (0.25%) + PABA (0.25%)	2	S–4B	4	42	59.5	9.9
Inositol (0.25%) + PABA (0.25%) + biotin (20 μg. %)	0	S–4B	5	42	127.8	21.3
Inositol (0.25%) + PABA (0.25%) + biotin (20 μg. %)	2	S–4B	5	42	93.8	15.6

[1] Some of these weights are expressed as averages, together with the standard error.

when tested alone, in synthetic rations containing succinylsulfathiazole: glutamine, indole-3-acetic acid, anthranilic acid, chondroitin sulfuric acid, gum arabic, calcium gluconate, and adenine.

In a survey of various natural products which were effective in promoting growth in a synthetic diet containing succinylsulfathiazole, powdered grass [5] appeared to have unusual activity. Since it was conceivable that the remarkable effect of proteose-peptone in promoting growth was due to its content of sulfonamide inhibitors (Lockwood and Lynch, '40), and to its ability to promote bacterial growth, rather than to any unusual content of essential dietary factors, a study of the effective factor or factors present in powdered grass was undertaken.

Heat stability

Fifty grams of powdered grass were mixed with 500 ml. of water and autoclaved at 15 pounds pressure for 1 hour. The mixture was filtered and the filter cake repeatedly washed with water. The combined filtrates were evaporated to dryness in a current of air on the steam bath. The activity was readily extracted by water and there appeared to be no heat destruction of the active materials. Extraction of powdered grass with water in a Waring blendor gave a less satisfactory yield of the active fraction.

Adsorption characteristics

The combined filtrates (from the extraction by autoclaving of 50 gm. of powdered grass with 500 ml. of water) were brought to pH 2 and stirred with 20 gm. of Darco G-60 charcoal for 30 minutes; this process was repeated with a 10 gm. portion of charcoal for 15 minutes. The filtrates were neutralized with sodium hydroxide and evaporated to dryness in a current of air on a steam bath. The effect of this extract on the growth rate of rats, on a diet containing 2% succinylsulfathiazole, indicated that the factor or factors involved were completely adsorbed on charcoal (table 5).

[5] Cerophyl.

Elution with barium hydroxide

The combined charcoal residues from the adsorption of powdered grass extract were stirred with four successive 250 ml. portions of saturated aqueous barium hydroxide. The combined eluates were neutralized with sulfuric acid and the filtrate evaporated to dryness. Barium hydroxide was ineffective as an eluting agent for the active material (table 5).

The failure to be eluted from charcoal by saturated barium hydroxide was taken as evidence of the dissimilarity of the factor or factors in question and the so-called G. P. 1 factor of Woolley ('42), which is readily eluted from charcoal by barium hydroxide. Microbiological assay indicated that "folic acid" was not eluted from charcoal by saturated barium hydroxide under the conditions employed.

Precipitation with lead acetate

The combined filtrates from the extraction by autoclaving of 50 gm. of powdered grass with 500 ml. of water were treated with an excess of saturated aqueous lead acetate solution. Excess lead was removed from the filtrate with hydrogen sulfide. The first lead precipitate was decomposed by trituration with excess sulfuric acid, and the lead sulfate removed by filtration. Both fractions were evaporated to dryness and fed as indicated in table 5. Activity was present in both portions and indicated the possible existence of two growth-promoting factors. Microbiological assay showed that 90% of the "folic acid" in powdered grass was present in the lead precipitated fraction.

Ether extraction

Fifty grams of powdered grass were extracted with anhydrous ether in a Soxhlet extractor until a colorless extract was obtained. The ether was evaporated and the unextracted residue was allowed to dry in the air. All the activity appeared to be retained in the unextracted portion (table 5).

TABLE 5

The effect of various 'Cerophyl' fractions on the growth of rats fed a diet containing 2% succinylsulfathiazole.[1]

SUPPLEMENT [2]	LEVEL OF SUCCINYL-SULFA-THIAZOLE	NUMBER OF ANIMALS	DURATION OF EXPERIMENT	AVERAGE GAIN IN WEIGHT	
				Total	Per week
	%		*days*	*gm.*	*gm.*
None	0	5	28	78	19.5
None	2	4	28	55	13.8
'Cerophyl'	0	6	28	96	24.0
'Cerophyl'	2	6	28	96	24.0
Room temperature extract of 'Cerophyl'	2	7	28	82	20.5
Autoclaved extract of 'Cerophyl'	2	6	28	81	20.2
Room temperature residue of 'Cerophyl'	2	6	28	94	23.5
Autoclaved residue of 'Cerophyl'	2	6	28	67	16.8
Room temperature extract and residue combined	2	5	28	90	22.5
Autoclaved extract and residue combined	2	5	28	82	20.5
None	0	5	42	131	21.9
None	2	5	42	77	12.8
'Cerophyl'	0	5	42	154	25.7
'Cerophyl'	2	5	42	121	20.2
'Cerophyl' extract	2	5	42	131	21.8
'Cerophyl' extract adsorbed at pH 2 with norite	2	8	42	81	13.5
$Ba(OH)_2$ eluate from charcoal after adsorption at pH 2	2	9	42	85	14.2
None	0	3	42	133	22.2
None	2	3	42	66	11.0
'Cerophyl'	0	5	42	149	24.8
'Cerophyl'	2	5	42	142	23.7
Lead acetate filtrate	2	5	42	96	16.1
Lead acetate precipitate	2	6	42	88	14.7
Alcoholic extract of 'Cerophyl'	2	6	42	83	13.8
Alcoholic residue of 'Cerophyl'	2	6	42	133	22.2
Ether extract of 'Cerophyl'	2	5	42	69	11.5
Ether residue of 'Cerophyl'	2	4	42	113	19.0

[1] Diet S–7 was employed.

[2] All supplements were tested at a level equivalent to 5% 'Cerophyl'.

Alcohol extraction

Fifty grams of powdered grass were percolated with absolute alcohol until a colorless extract was obtained. The alcohol was evaporated on the steam bath and the unextracted residue dried in the air. All the activity appeared to be retained in the unextracted portion (table 5).

The occurrence of marked "anti-succinylsulfathiazole" activity in dried grass, the ease of adsorption of activity on charcoal, the failure to be eluted with barium hydroxide, the precipitation by lead acetate, and the insolubility of the material in alcohol and ether, all pointed to the possibility of one of the factors being "folic acid." At this stage of the investigation a personal communication from Dr. C. A. Elvehjem indicated that he had found "folic acid" remarkably effective in promoting growth in a ration containing succinylsulfathiazole. Biotin was found by him to accentuate the growth-promoting effect of "folic acid."

A concentrate of "folic acid" (200,000 Snell-Peterson units per gram) and either a biotin concentrate (SMA no. 200) or crystalline biotin were administered orally by stomach tube daily to rats which had reached a plateau in their growth rate on a synthetic ration containing succinylsulfathiazole (2%). In our hands "folic acid" and biotin, when administered singly, each has some activity in overcoming the growth depression caused by succinylsulfathiazole; however, when these substances are administered together a striking stimulation of growth results (table 6).

In animals fed over several weeks on purified diets containing added succinylsulfathiazole occasional spontaneous bleeding has occurred; sometimes the animals succumbed to an acute blood loss but usually they survived. The prothrombin times have been determined occasionally on experimental animals. In one series of determinations results were obtained as indicated in table 7. Animals receiving only the basal ration containing succinylsulfathiazole had a definite prolongation of the prothrombin time. Supplements of either

TABLE 6

The effect of feeding "folic acid" and biotin to rats fed a diet containing 2% succinylsulfathiazole.

EXPERIMENT NUMBER	DAILY SUPPLEMENT	NUMBER OF ANIMALS	GAIN IN WEIGHT			
			1st week	2nd week	3rd week	3-week period
			gm.	*gm.*	*gm.*	*gm.*
1[1]	None	9	+2	−2	−7	−7
1	2 μg. biotin[3]	4	+2	+2	+2	+6
1	10 mg. "folic acid" concentrate[4]	2	−3	+5	+2	+4
1	2 μg. biotin[3] and 10 mg. "folic acid" concentrate[4]	5	+14	+11	+11	+36
2[2]	None	3	+2	−6	0	−4
2	5 μg. biotin[3]	2	+13	+9	+13	+35
2	20 mg. "folic acid" concentrate[4]	3	+4	+13	+15	+32
2	5 μg. biotin[3] and 20 mg. "folic acid" concentrate[4]	2	+23	+18	+18	+59
2	133 mg. grass juice powder[6]	4	+8	+17	+11	+36

[1] All animals in experiment 1 were depleted on ration S–7 for 7½ weeks before supplements were started.

[2] All animals in experiment 2 were depleted on ration S–7 for 9 weeks before supplements were started.

[3] SMA biotin concentrate no. 200, 20 μg. per milliliter.

[4] "Folic acid" concentrate 200,000 S–P units per gram.

[5] Crystalline biotin (Merck).

[6] 133 mg. grass juice powder equivalent in "folic acid" to 20 mg. "folic acid" concentrate.

biotin or of the "folic acid" concentrate alone were effective in decreasing the prothrombin time. The concentrate of "folic acid" and biotin together appeared to be superior to either factor alone in restoring the prothrombin time to normal.

In addition to growth inhibition, certain changes in the appearance of the rats were described earlier in this communication. A favorable effect on these changes was exerted by a combination of the "folic acid" concentrate and biotin and occasionally by one or the other factor alone.

TABLE 7

The prothrombin time of rats receiving 2% succinylsulfathiazole [1] *and various dietary supplements.*

GROUP NUMBER	DAILY SUPPLEMENT	NUMBER OF ANIMALS	DURATION OF DEPLETION	DURATION OF SUPPLEMENT FEEDING	PROTHROMBIN TIME [2]
			days	*days*	*seconds*
1	None	3	79	0	> 240, 134, > 180
			Average		*> 185*
2	5 μg. biotin	3	63	16	105, 60, 55
			Average		*73*
3	20 mg. "folic acid" concentrate	3	63	16	46, 70, 46
			Average		*54*
4	5 μg. biotin and 20 mg. "folic acid" concentrate	4	63	16	49, 46, 42, 42
			Average		*45*
5	133 mg. grass juice powder	4	63	16	45, 54, 41, 45
			Average		*46*

[1] Diet no. S–7 was employed.

[2] Citrated plasma diluted 1: 8 with saline (0.1 cc.); freshly restored lyophilized rabbit brain thromboplastin (0.1 cc.); calcium chloride, 0.277% solution (0.1 cc.); all tests run at 37°C.

DISCUSSION

The effect of ingested succinylsulfathiazole on the number and type of organisms present in the intestinal tract of the rat has not yet been studied extensively. In the dog succinylsulfathiazole induces a striking reduction in the coliform count of the feces (Poth and Knotts, '41). However, studies in monkeys indicated that, under the experimental conditions which prevailed, where the predominating flora were made up of lactobacilli, streptococci and anaerobic bacteria, succinylsulfathiazole had a less marked effect on the initially low coliform count of the feces (Welch et al., '42). Apparently succinylsulfathiazole affects primarily the coliform organisms (vitamin synthesizers) and leaves more or less uninfluenced the lactobacilli, streptococci, and anaerobic bacteria (vitamin requirers). Points still to be determined are the extent to

which "folic acid" and biotin promote the synthesis, by the residual intestinal bacteria, of additional essential dietary factors, and the extent to which "folic acid" and biotin are required by the tissues of the rat. Supplementing a succinylsulfathiazole ration with either "folic acid" or biotin decreased the prolonged prothrombin time of the rats to a value approaching that of normal animals, an observation which strongly suggests that a stimulation of the synthesis of vitamin K by intestinal bacteria occurred (Greaves, '39). A combination of "folic acid" and biotin, both of which are required as growth factors by many species of lactobacilli, streptococci (Snell and Peterson, '40; Mitchell et al., '41; Hutchings et al., '41) and anaerobic bacteria (Mueller and Miller, '42), restored the prothrombin time to that found in animals which were given a grass juice supplement high in vitamin K. These findings indicate that at least part of the effect produced by these factors is to promote a certain type of bacterial synthetic activity. However, "folic acid" and biotin might be involved in the utilization of vitamin K by the rat. Presumably biotin and "folic acid" are essential components of certain metabolic systems of the rat; however, further investigation will be required to elucidate the mechanism of action of these substances. Concerning biotin, the sum-total of all available evidence is suggestive of a direct role of this factor in animals; in the case of "folic acid" preliminary evidence supports the same conclusion. Data are accumulating which indicate that succinylsulfathiazole may influence the intestinal synthesis of still other factors which are concerned with growth or other physiological processes in the rat.

Extensive pathological changes in the tissues of rats given either sulfaguanidine or succinylsulfathiazole have been described (Daft, Ashburn and Sebrell, '42; Daft, Ashburn, Spicer and Sebrell, '42). Although growth changes have occupied our particular attention, careful histological study was made of the tissues of two large series of rats given succinylsulfathiazole. In the first series inositol and p-amino-

benzoic acid were not given (S-1); in the second series the basal diet (S-7) contained these compounds. Suffice it to say that in neither case have we been able to find definite changes of an intensity comparable to those described by Daft and Sebrell. No simple explanation of the discrepancy in findings is available at present.

Black et al. ('42) showed that the effects of sulfaguanidine can be markedly antagonized by the administration of PABA, either orally or parenterally. In our experience with succinylsulfathiazole the effect of this sulfonamide-antagonist has not been striking. It will be noted that marked inhibition of growth is produced by succinylsulfathiazole when both PABA and inositol are present in the diet (tables 4 and 5). Extensive study of the effect of the drug in the presence of PABA, but in the absence of inositol, has not been made; however, the effect of 0.2% PABA added to diet S-1 (no inositol) indicates that a slight effect was exerted. Additional attention will be given to this point.

Theories concerning the mechanism of action of succinylsulfathiazole have, for the most part, revolved around the concept of a gradual liberation of sulfathiazole in the intestine, in amounts insufficient to produce a significant concentration of sulfathiazole in the blood and other tissues. Such a splitting might result in a localized bacteriostatic concentration of the sulfonamide (Poth et al., '42; Kirby and Rantz, '42). The possibility should not be overlooked, however, that succinylsulfathiazole per se may have some specific effect on certain enzyme systems.

SUMMARY

The inhibition of growth and increase in prothrombin time brought about by the inclusion of succinylsulfathiazole in highly purified rations adequate in the well-recognized members of the vitamin B complex could be largely overcome by supplements of a "folic acid" concentrate and crystalline biotin.

The relationship of "folic acid" and biotin to bacterial activity in the intestine is discussed.

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THE BIOLOGICAL ASSAY OF PYRIDOXINE (VITAMIN B_6)

MIRIAM F. CLARKE AND MARIE LECHYCKA

The Burroughs Wellcome & Co., U. S. A., Experimental Research Laboratories, Tuckahoe, New York

TWO FIGURES

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Until a chemical or microbiological method for assay of substances containing pyridoxine has been accepted as a satisfactory and reliable procedure, recourse must be had to a biological method for comparative purposes. With this in mind, the present study was undertaken to establish procedures for the assay and to determine what accuracy could be expected by its use.

Two methods in which the rat is the test animal have been widely employed in the bioassay of pyridoxine, one involving the cure of typical acrodynia, and the other increase in weight with graded doses of the vitamin. The curative procedure was not adopted for several reasons as follows: (1) vitamin deficiencies other than pyridoxine may influence occurrence and cure of rat dermatitis; (2) severity of dermatitis may vary with the season; and (3) judgment of the severity of dermatitis as well as the extent to which a cure has been effected may be seriously open to subjective influences. However, since satisfactory results have been obtained using graded doses of pure pyridoxine (Dimick and Schreffler, '39; Conger and Elvehjem, '41) in relation to gain in weight over a period of weeks, the growth method was chosen. Moreover, it has been shown (Gaddum, '33) that "if the response can be measured quantitatively it will only be necessary to use

about half as many animals to attain a given degree of accuracy as would be necessary if the experimental data only indicated the proportion of animals in which the response exceeded some fixed threshold." In our experience the threshold, which in the case of pyridoxine is the complete cure of symptoms, is not easily determined with exactness.

EXPERIMENTAL

Albino rats maintained in our colony on a uniform ration [1] were employed in this study. When the young were 16–19 days old and weighed between 25 and 40 gm. they were placed with their mothers on one of the B-complex free rations described below. This early transfer to the depletion diet was adopted to shorten the time required to deplete the young rats by limiting the time in which they had access to a rich food supply. At weaning, on the twenty-first day, the young were placed in individual cages with raised screen floors. They were continued on the basal ration with the water-soluble supplements administered separately either six times weekly (double dose on the day preceding a Sunday or holiday) or, later, three times weekly as explained in the test. With the exception of pyridoxine the supplements were started at weaning. Ten to twelve rats were assigned to each group, the groups within an experiment being similar with regard to sex, weight, litter representation and number of rats.

Two diets have been used (table 1). Preliminary work showed that rats on diet 11, supplemented with sources of the water soluble vitamins except pyridoxine, were depleted in approximately 7 weeks. In diet 12 changes were introduced which affected mainly the fat content. In all work with diet 12, which was used throughout the experiments to be discussed, and which involved the use of several substances to provide the so-called "filtrate factors," the depletion time averaged 5 weeks.

[1] Purina fox chow; lettuce three times weekly. Some of the litters were from breeders given supplements to this diet. No difference in the performance of the latter was discernible.

Dietary supplements for pyridoxine assay rats

The first part of this study is concerned with finding a suitable supplement which would supply all of the water-soluble vitamins in adequate amounts for the rapidly growing white rat, but which would be relatively free of pyridoxine so that rats would be depleted of this vitamin within a reasonable time. It seemed desirable to supply thiamine, riboflavin, nicotinic acid and choline as pure substances, supplementing them with a natural concentrate containing pantothenic acid and other factors required by the rat. For this purpose both yeast preparations (Kuhn and Wendt, '38; Chick et al., '40), and liver filtrates (Dimick and Schreffler, '39; Weigand et al., '40; McElroy and Goss, '40 b; Emerson, '41; Conger and Elvehjem, '41; Lepkovsky and Krause, '42; and Cerecedo and Foy, '42) have been employed. Likewise, a rice polishings preparation, free of vitamin B_6 (Supplee et al., '40) has been successfully used. A rice polishings concentrate [2] was chosen because of its uniformly high pantothenic acid content (400 μg. per gm.). It also has a high content of choline (du Vigneaud et al., '39) which is a dietary essential for the rat. Moreover, in riboflavin assays, the same preparation had been successfully employed for the same purpose (Clarke et al., '40).

TABLE 1

Diets used to produce pyridoxine deficiency.

	DIET 11 [1]	DIET 12 [1]
	%	%
Casein, Labco or Smaco brands	22	22
Sucrose, U.S.P.	68	69.5
Salt mixture, U.S.P. no. 1	4	4
Hydrogenated cottonseed oil [2]	4	2
Cod liver oil, U.S.P.	2	2
Wheat germ oil	—	0.5

[1] Diets were supplemented with water-soluble vitamins, given separately.
[2] Crisco.

[3] "Ryzamin-B," Burroughs Wellcome and Co. (U. S. A.) Inc.

To remove the vitamin B_6 present in the rice polishings concentrate, a 900 gm. lot was diluted so that the resulting solution contained 5% solids. The pH was adjusted to 4.0. The solution was treated with 338 gm. of fuller's earth, stirred for 3 hr., allowed to settle, siphoned off, centrifuged and filtered. This process was repeated five times. The final solution, after filtering, was evaporated in a partial vacuum at 50°C. to a heavy syrup of sp. gr. 1.43. The yield was 590 gm. This product is referred to as "treated rice polishings concentrate." It was fed as a daily supplement of 75 or 150 mg. together with the vitamins mentioned previously: thiamine 48 μg., riboflavin 40 μg., nicotinic acid 20 μg. and choline chloride 2 mg.

The treated concentrate was also compared with a similar commercial preparation.[3]

All vitamins given in addition to, or in place of, the concentrates from rice were pure except biotin which was a commercial preparation containing 100 μg. biotin per cubic centimeter.[4]

The rats were weighed three times weekly until they were depleted. Preliminary studies had shown that symptoms appeared at about the time when the growth rate was becoming very slow. The symptoms considered characteristic for pyridoxine deficiency were those of the ears, paws and mouth (snout and lips) described by Supplee et al. ('40), and in addition tail symptoms similar to those observed by McElroy and Goss ('40 a). If a rat had any one of these symptoms and was stationary in weight it was considered depleted and the supplement, pyridoxine or a concentrate containing this vitamin, was then administered in daily doses for 4 weeks. A rat which is stationary in weight is defined as one which (a) weighs the same as or less than it did 1 week before, (b) has all weighings for 1 week agreeing within 3 gm., or (c) has lost 3 or more grams in two weighing periods, i.e., in 4 or 5 days.

[3] Labco brand, rice polish factor II, The Borden Co.

[4] S. M. A. Corp., biotin concentrate no. 1000.

RESULTS

Comparison of supplements

Two criteria were used in deciding whether the supplements would be satisfactory for vitamin B_6 assay purposes: (a) time required for depletion, and (b) gain in weight when either a maximum dose of pyridoxine, or pyridoxine plus an untreated extract of rice polishings (table 2) was given.

The two preparations from rice polishings were each used at 75 mg. and 150 mg. levels (table 2, expts. 1–4). Fairly uniform results on time required for depletion seem to indicate that these two substances are reasonably free of vitamin B_6, allowing depletion in approximately 31 days. During the assay period, gain in weight when a daily dose of 50 μg. pyridoxine was added was less when a 75 mg. dose of either rice polishings preparation was employed than when 150 mg. was fed throughout the experiment as the basal supplement. Evidence that even the 150 mg. dose of these preparations was lacking in some factor was obtained when, in addition to 50 μg. pyridoxine, 150 mg. per day of untreated rice polishings concentrate was given during the 4 week assay period (expts. 1 and 3). This suggests that the treatment of the concentrate to remove pyridoxine had resulted in the loss of some other substance also required by the rat. Microbiological assay (Pennington et al., '40) of the rice polishings concentrate, before and after the adsorption treatment, revealed the fact that the pantothenic acid content had been reduced to about one-third its original value. Experiment 5, in which the pantothenic acid of the concentrate was restored, shows that the growth rate was the same in the assay period as when 150 mg. of the untreated product had been employed (expt. 1); namely, about 120 gm. in 4 weeks, a figure which approaches the maximum for our strain of rats. Adding 1 μg. biotin per rat per day in the form of a concentrate produced a slight but insignificant increase in gain (expt. 6).[5]

[5] Comparing gains in weight, expt. 5 and expt. 6, $t = 0.96$. For $n = 21$, the statistic t corresponds to a probability, P, greater than 0.05, and the difference is not significant.

TABLE 2

Criteria used in judging whether sources of water-soluble vitamins, supplementary to diet 12, were adequate for B_6 assay rats.

EXPT. NO.	SUPPLEMENT GIVEN THROUGHOUT DEPLETION AND ASSAY PERIODS [1]	TIME REQUIRED FOR DEPLETION	GAIN IN WEIGHT 4 WK. ASSAY PERIOD	
			Dose: 50 μg. pyridoxine daily	Dose: 50 μg. pyridoxine + 150 mg. untreated rice polishings concentrate daily
		days	*gm.*	*gm.*
1	75 mg. treated rice polishings concentrate + 2 mg. choline	30.9 ± 1.4 [2]	74.5 ± 3.9 [2]	118.9 ± 7.2 [2]
2	150 mg. treated rice polishings concentrate + 2 mg. choline	31.8 ± 2.4	107.4 ± 6.6	————
3	75 mg. filtrate factor II [3] + 2 mg. choline	29.4 ± 1.3	65.8 ± 4.3	120.9 ± 7.4
4	150 mg. filtrate factor II + 2 mg. choline	32.4 ± 1.5	92.4 ± 4.6	————
5	150 mg. treated rice polishings concentrate + 2 mg. choline + 60 μg. calcium pantothenate	32.0 ± 1.1	121.6 ± 5.8	————
6	150 mg. treated rice polishings concentrate + 2 mg. choline + 60 μg. calcium pantothenate + 1 μg. biotin [4]	31.3 ± 2.7	130.0 ± 6.5	————
7	5 mg. choline + 110 μg. calcium pantothenate + 1 μg. of biotin	30.0 ± 1.5	123.6 ± 9.4	————

[1] Each rat received daily, in addition, 48 μg. thiamine, 40 μg. riboflavin and 20 μg. nicotinic acid.

[2] Standard error, $S\bar{y} = \sqrt{\frac{S(y-\bar{y})^2}{n(n-1)}}$

[3] Labco.

[4] Biotin concentrate no. 1000, S. M. A. Corp., contains 100 μg. biotin per cubic centimeter.

At this point it seemed obvious that the preparations made from rice polishings were not supplying much that could not be furnished by purified preparations. Moreover, Unna ('40 a) and others have obtained satisfactory growth and freedom from symptoms in rats receiving only pure vitamin supplements. Consequently, the treated rice polishings concentrate was omitted entirely, the choline and calcium pantothenate increased to make up for its removal, and biotin included (the only non-purified material); the resulting growth came up to the maximum standard for our rats (expt. 7). Depletion time for this group, including time of appearance of pyridoxine deficiency symptoms, was similar to all the others.

Specificity of the symptoms of the rats

Examination of the symptoms of vitamin B_6 deficient rats maintained on diet 12 with purified supplements indicated that pure pyridoxine or concentrates containing this vitamin fed during an assay period cured all lesions except those of the tail. With larger doses of B_6 a proportionately greater percentage of rats was cured in the 4-week period, but there was no tendency for larger doses to cure the symptoms in a shorter time, the average time of cure for fifty rats in one experiment being 18.8 ± 7.3 (standard deviation) days for doses of 1 to 50 μg. daily. Cure of the symptoms by pure pyridoxine offers support to the conclusion that our rats were depleted of this vitamin only.

Rats receiving 80 μg. of pantothenic acid (Unna, '40 b) and 10 mg. linoleic acid (Quackenbush et al., '42) as prophylactic doses should remain free of skin symptoms associated with a deficiency of these substances. The former is supplied in the supplement; the latter is contained in diet 12 both in the hydrogenated cottonseed oil,[6] in limited amounts,[7] and in the wheat germ oil (Sullivan and Bailey, '36).

[6] Crisco.

[7] Personal communications from Dr. George O. Burr, University of Minnesota, and Mr. H. S. Brutton, The Procter & Gamble Company.

Symptoms of the tail were observed in many rats. These ranged from slight swelling or scaliness at the tip to severe swelling with exudate involving most of the tail. Lesions of the tail sometimes became much more severe immediately after the vitamin B_6 dose was started, especially with the larger amounts as noted by McElroy and Goss ('40 a). Cures followed as the vitamin administration proceeded. However, when lesions were severe with exudate, swelling and necrotic rings, complete cures were not effected. In these cases the end of the tail frequently dropped off leaving a scarred stump. It seems probable that the tail symptoms are specific for vitamin B_6 deficiency but, unlike the other dermal symptoms, they are associated with pathological changes so severe as to be irreversible by dosing with pyridoxine.

The dose-response curve

Since specific pyridoxine deficiency symptoms and satisfactory growth responses were obtained when purified supplements were used, the latter were employed with diet 12 for establishing the dose-response relationship in bioassays. Two further changes were introduced: (1) the addition of inositol, suggested (Pavcek and Baum, '41; Gavin and McHenry, '41) as a requirement of the rat; and (2) the arrangement of administration of the basal supplement on 3 days instead of 6 to reduce labor. The following supplement was given in 1 cc. of solution on Mondays, Wednesdays and Fridays throughout depletion and assay periods: thiamine hydrochloride 100, riboflavin 100, nicotinic acid 50, choline chloride 12,000, inositol 12,000, calcium pantothenate 225 and biotin 2 μg., respectively.

Depletion time, using this supplement and method of dosing, was somewhat longer than that previously mentioned for other supplements, 42 ± 1.2 (standard error) days for the males and 40 ± 1.4 days for the females. The time required for depletion may have been influenced by the season, most of these rats having been on experiment in late spring and early summer when appearance of symptoms seems to be somewhat

delayed. The average weight at the start of the assay was 87 gm. for males, and 80 gm. for females.

Preliminary work had suggested that, within limits, the logarithm of the dose of pyridoxine might bear a linear relation to the response expressed as gain in weight in 4 weeks. Doses were selected accordingly and were fed daily to groups of ten rats, five males and five females. The results are plotted in figure 1. A range of dose from 1 to 8 μg. produces

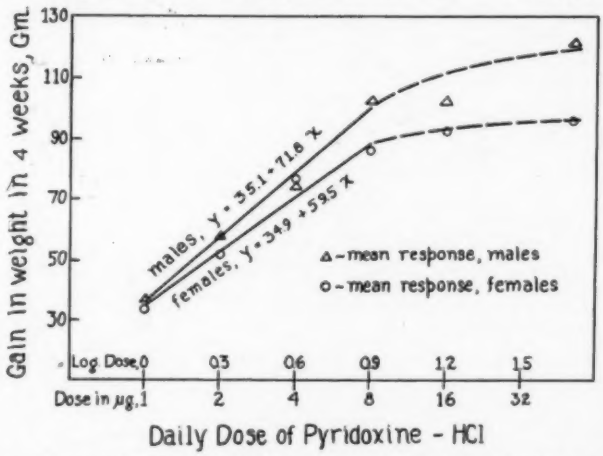
Gain in weight in 4 weeks, Gm.
130
110
90
70
50
30
males, Y = 35.1 + 71.8 x
females, Y = 34.9 + 59.5 x
△-mean response, males
○-mean response, females
Log Dose 0 .3 .6 .9 1.2 1.5
Dose in μg. 1 2 4 8 16 32
Daily Dose of Pyridoxine - HCl

Fig. 1 Curves relating graded doses of pyridoxine hydrochloride to gain in weight of rats in 4 weeks. The two highest doses, 16 and 50 μg., were not used in calculating the equations for the regression lines.

responses between approximately 30 and 90 gm., each 100% increase in the dose producing an additional 20 gm. gain in the males, and somewhat less in the females. Doses larger than 8 μg. produce gains that approach the maximum but which are not on the linear portion of the graph. The maximum response for the females is lower than that for the males. The equations representing the dose-effect curves are:

$$y = 35.1 + 71.8\,x \text{ (males)},$$
$$y = 34.9 + 59.5\,x \text{ (females)},$$

where y is the gain in weight in 4 weeks and x is the logarithm of the dose in micrograms. Using methods outlined by

Snedecor ('40), it has been shown that the linear relation holds from a dose of 1 μg. to 8 μg. and that no significant difference in slope for males and females has been established in this experiment.

A bioassay

An assay for pyridoxine potency of rice polishings concentrate is described to illustrate the use of the information set forth above. Three dose levels, at equal logarithmic intervals,

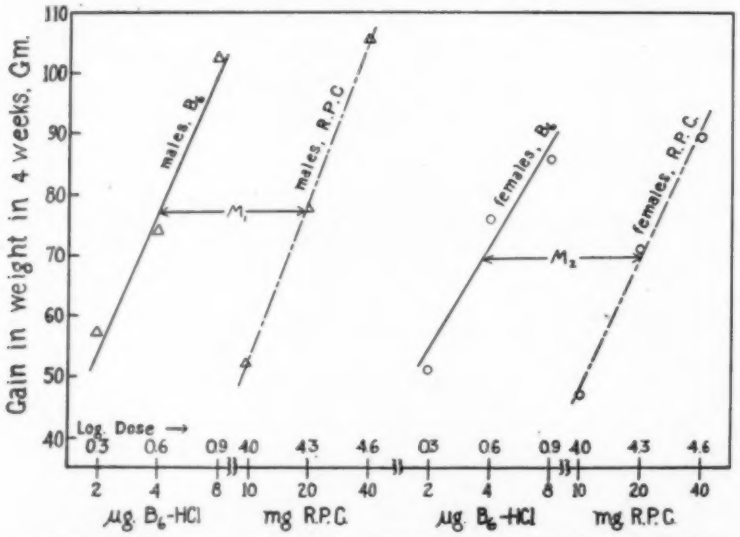

Fig. 2 Graphic representation of a bioassay of rice polishings concentrate. M, log ratio of potency $= \bar{x}_s - \bar{x}_u$, where $\bar{x}_s$ is the mean (approximate) log dose for the standard, $\bar{x}_u$ is the corresponding mean log dose of the unknown (for the same response level). Both values are read from the abscissa. For males, antilog $M_1 = 214$ in terms of μg./gm. For females, antilog $M_2 = 178$ μg./gm.

were employed for both the unknown and the standard. For the standard, 2, 4 and 8 μg. of pure pyridoxine hydrochloride were used; for the unknown, 10, 20 and 40 mg. were selected. The graph (fig. 2) shows the results for males and females plotted separately and suggests the method of arriving at the

potency by means of the term M, the log ratio of potency, which can be found by an entirely graphic method. To obtain a more accurate estimate of potency and to find the error of the assay, analyses of variance and polynomial coefficients were made by the method described by Morrell ('42) for thiamine assays on the rat in which a balanced design of experiment was used also. In the bioassay described here, the potency found was 203 (range 158–260) μg. per gram, based on male data; 188 (range 158–224) μg. per gram, based on female data.

Accuracy obtained

The limits of error which determine the range are for a probability of 0.05 and represent the range in which the potency would occur in twenty-one trials out of twenty-two.

TABLE 3

Effect of length of assay period and sex of rats on accuracy of the bioassay.

NUMBER AND SEX OF RATS	4 WEEKS			3 WEEKS			2 WEEKS			1 WEEK		
	Potency	Limits [1]	Standard error of gain [2]	Potency	Limits [1]	Standard error of gain [2]	Potency	Limits [1]	Standard error of gain [2]	Potency	Limits [1]	Standard error of gain [2]
	μg./gm.	%	gm.	μg./gm.	%	gm.	μg./gm.	%	gm.	μg./gm.	%	gm.
30 ♂	203	78–128	11.8	195	80–125	9.1	173	79–127	7.7	174	74–135	5.2
30 ♀	188	84–119	6.3	191	84–119	5.9	198	82–122	5.1	169	69–145	4.7

[1] Limits for $P = 0.05$, expressed as per cent of potency.

[2] Standard error of estimate determined from the mean square for error in the analysis of variance.

The experimental error in this assay is somewhat less for the females than for the males. For practical reasons, the potency and error were calculated for assay periods of 3, 2 and 1 week periods as well as for 4 weeks (table 3). The results indicated that the accuracy obtainable from the 3 and 2 weeks' calculations is close to that when a 4-week period was used. In 1 week the error is somewhat larger. The limits of error for females are less than for males in the 4, 3 and 2 week

assay periods. Examination of the standard errors for gain in these three intervals shows that the error for females is considerably smaller than the corresponding value for males. Application of the Z test (Fisher and Yates, '38) indicates that the difference is significant.[8] The larger standard error for males more than compensates for the greater slope of the dose-response curve and the larger absolute gains for males, both of which would tend to make the experimental error smaller. These findings indicate that females should be used instead of males for bioassays of vitamin B_6 under our conditions.

SUMMARY

1. A diet and supplements suitable for pyridoxine assay by means of growth of the rat have been described.

2. Tests showed that the supplement selected, consisting entirely of pure vitamins except for biotin, was adequate for the purpose.

3. Symptoms of the rats are shown to be specific for pyridoxine deficiency, the tail lesions being somewhat unique.

4. The dose response curves for males and for females are found to be linear from doses of 1 μg. to 8 μg. when the logarithm of the dose is plotted against the response expressed as gain in weight in 4 weeks; the slope of the curve for females is slightly less than that for males.

5. Details of the arrangement of the assay and a method of analysis of results to find potency and error are suggested.

ACKNOWLEDGMENT

The authors are grateful to Dr. Charles A. Cook for preparation of the rice polishings concentrate and to Miss Sylvia Charlow for assistance in animal experiments.

[8] The statistic Z corresponds to a probability, P, less than 0.05 when the variance of gain in weight of females is compared with that of males for the 2, 3 and 4 week assay periods. The differences may be considered significant.

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THE CONTRIBUTION OF NON-FAT MILK SOLIDS TO THE NUTRITIVE VALUE OF WHEAT BREADS[1]

H. H. MITCHELL, T. S. HAMILTON AND J. B. SHIELDS
WITH THE TECHNICAL ASSISTANCE OF J. R. BEADLES
Animal Nutrition Division, University of Illinois, Urbana

THREE FIGURES

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The nutritive deficiencies of patent white flour, and of the bread made from it with no nutritive supplements, have been so widely publicized as to need no further comment. However, the conception thus impressed on the public mind that bread is deficient in the essential nutrients would seem to apply more to the bread formula used many years ago than to modern commercial bread. For example, Prouty and Cathcart ('39) have shown that, due to the incorporation of relatively large amounts of dried skim milk and calcium salts as yeast foods, the most popular brands of commercial breads today contain an average of almost three times the calcium content of 0.03% frequently cited as representative of bread; even the lowest per cent calcium in these modern breads exceeds this value, as well as the average calcium content of the whole wheat berry itself expressed on a comparable moisture basis. Furthermore, Fairbanks ('38, '39) has shown that the addition of 6% of skim milk powder (on the basis

[1] The prosecution of this research was aided by the donation of funds to the University of Illinois by the American Dry Milk Institute, Inc., Chicago, Ill. Grateful acknowledgment is also made to the Institute, and particularly to Mr. Charles O'Malley, Mr. Harry G. Brouilett, and Mr. C. A. McDuffee, for their aid in the preparation of the experimental breads and in the vitamin analysis. Except for the actual baking of the bread, these operations were supervised by one of the authors (J.B.S.).

of the white flour) to white bread markedly improves the growth-promoting value of the bread and the rate of bone calcification, as revealed by feeding experiments on growing albino rats. Henry and colleagues ('41) have shown in carefully controlled experiments on growing rats that this improvement in the nutritive value of white bread brought about by skim milk powder is traceable to an increase in the amount and quality of the protein, and in the content of calcium, riboflavin and possibly other members of the B_2-complex.

Recently, a nation-wide campaign for the enrichment of white flour and bread with thiamine, iron, nicotinic acid and ultimately riboflavin, has been initiated, a campaign motivated by the conviction held in influential quarters that the American diet is quite generally inadequate in many respects, but prominently in thiamine. A standard for enriched flour was promulgated on May 27, 1941, by the Federal Food and Drug Administration, and a proposal for a definition and standard for enriched bread was issued by the same agency on June 7, 1941. The nutritive advantages of thiamine supplements to white bread have been studied by Pewters, Mason and Higgins ('41) and by Free ('40), on rats and human subjects, respectively, with results that were to be expected. Whether such supplementation will improve 73% extraction flour to the extent that its growth-promoting power equals that of the national wheat meal of Great Britain, representing a mill extraction of 85%, has been studied with conflicting results by Chick ('40) and by Wright ('41). The experiments of Wright, indicating no difference between the two flours, were much better controlled than those of Chick.

The enrichment of flour and bread with thiamine, iron and nicotinic acid according to the prevailing practice is undoubtedly a wise procedure that may be expected to improve the nutritional status of a considerable, though quite unknown, proportion of the population. It would be regrettable, however, if this latest program for the nutritive betterment of bread should supersede the much older program of the inclusion of non-fat milk solids in bread, or should be con-

sidered the equivalent of such inclusion.[2] The experiments to be described in this paper were designed to assess the value of skim milk powder as a white bread adjuvant, not only in comparison with the enriching nutrients of the official program, but also in comparison with those portions of the wheat kernel discarded in the milling of patent white flour. We were also interested in discovering the extent to which enriched bread and whole wheat bread would themselves be improved nutritively by the inclusion of dried skim milk, if such improvement occurred at all.

EXPERIMENTAL PROCEDURES

Three series of comparisons were made with young albino rats, each series involving three different kinds of bread. Most of the breads used were made with a white patent flour [3] representing 70 to 75% of the whole wheat berry, depending upon the weight of the wheat and the character of the bran. The whole wheat bread was made from a flour [4] representing the entire wheat berry. All breads were baked at 425 to 435°F. for 30 to 40 minutes. They were then sliced, dried at 140°F. and ground to a powder. The essential features in the description of the test breads are as follows:

Series 1. Bread A — White flour, no milk solids, enriched with fortified yeast. Bread B — White flour, 6 parts of dried skim milk per 100 parts of flour (6%), unfortified yeast. Bread C — White flour, 6% dried skim milk, enriched with fortified yeast.

Series 2. Bread D — Whole wheat flour, no milk solids, unfortified yeast. Bread E — White flour, no milk solids, enriched with fortified yeast, dicalcium phosphate and ribo-

[2] The reality of the danger that the contribution of milk solids to the nutritive value of bread may be overlooked is illustrated by a recent report of the Council on Foods and Nutrition of the American Medical Association on the present nutritional status of flour and bread. In this report (J. Am. Med. Assoc., vol. 116, pp. 2849–2853 (1941)) no mention is made of the presence of milk solids in modern bread or of their contribution to its nutritive value.

[3] Pillsbury's Pan-King Flour.

[4] Procured from the Elam Milling Co.

flavin to equal bread F. Bread F — White flour, 6% dried skim milk, enriched with fortified yeast.

Series 3. Bread G — Whole wheat flour, no milk solids, unfortified yeast. Bread H — White flour, 6% dried skim milk, unfortified yeast. Bread J — Whole wheat flour, 6% dried skim milk, unfortified yeast.

The dried breads were fed as the sole diet to growing rats, except for a supplement of fortified cod liver oil given to all rats to provide vitamins A and D, in which all breads are deficient. The three breads of series 1 were compared by feeding them to ten trios of rats by a method of equalized feeding, each rat in each trio receiving one of the test breads and all rats in each trio receiving the same weight of dry matter.[5] They were compared also by feeding to ten other trios of rats, each rat in each trio receiving one of the test breads, but all rats consuming their respective diets ad libitum.

The three breads of series 2 were tested in a similar manner, except that twelve trios of rats were fed by the equalized food method, and eleven trios by the ad libitum method.

The three breads of series 3 were compared by feeding them to twelve trios of rats by a method of controlled feeding, according to which each rat in each trio received one of the test breads and all rats in each trio received such amounts of their respective diets as to promote equal gains within the trio.

The rats were started on the experiment at body weights of 40 to 45 gm., the experimental trios being equalized with reference to sex, initial weight and litter membership. They were weighed weekly and were continued on experimental feeding for about 12 weeks, or until the more rapidly growing members of a trio had gained 100 gm. or somewhat more in body weight. Samples of blood were taken from the tail and the rats then killed with ether and the body lengths from tip of nose to root of tail measured. The concentration of blood

[5] The moisture content of each batch of each bread was determined for this purpose.

hemoglobin was determined by the Sanford and Sheard ('30) method adapted to rat blood. Selected bones (femurs and humeri) from each carcass were taken for ash determinations on the dry, fat-free (ether-extracted) basis. Some of the carcasses in each experimental group of rats were analyzed for total iron by the Stugart ('31) modification of the Kennedy ('27) method, the dry ashing of the entire carcass being carried out at a temperature of 400°C. or less. Color comparisons were made with the Cenco-Sheard-Sanford photelometer. Some of the carcasses in each group were frozen, ground and analyzed for thiamine by the method of Hennessy and Cerecedo ('39), for riboflavin by the method of Hodson and Norris ('39), and for nicotinic acid by the microbiological method of Snell and Wright ('41).

The breads were analyzed for the usual constituents by standard methods of analysis, and the breads, as well as some of their components, for thiamine, riboflavin and nicotinic acid by the methods referred to above. The possible losses in thiamine and riboflavin during bread baking and drying were studied by analyzing (1) the dough, (2) the crust and crumb of the baked bread, and (3) the dried bread.

EXPERIMENTAL RESULTS

The composition of the breads. The dried skim milk used in the bread formulas contained 3.7 μg. of thiamine and 21.7 μg. of riboflavin per gram; the white flour, 0.66 and 0.38 μg. per gram, respectively; and the whole wheat flour, 3.7 and 1.0 μg. per gram, respectively. The analytical results obtained on the nine experimental breads are summarized in table 1. The enriched white water bread A contained per pound, on a moisture basis of 38%, 15.2 mg. of iron, 1.25 mg. of thiamine, 9.86 mg. of nicotinic acid and 0.21 mg. of riboflavin. It therefore falls within the limits of the proposed standard for enriched bread[6] except for the specification for riboflavin,

[6] According to the Federal Register for June 7, 1941, the proposed standard specifies the following lower and upper limits in the amounts of nutrients per pound of bread: 1 and 4 mg. of vitamin B_1, 0.8 and 3.2 mg. of riboflavin, 4 and 16 mg. of nicotinic acid or its amide, 4 and 16 mg. of iron, with optional limits of 160 and 640 U.S.P. units of vitamin D and 333 and 1333 mg. of calcium.

TABLE 1

Chemical composition of the experimental breads.

BREAD	DESCRIPTION OF BREAD	MOISTURE	CRUDE PROTEIN	ETHER EXTRACT	CRUDE FIBER	GROSS ENERGY	ASH	CALCIUM	PHOSPHORUS	IRON	THIAMINE	NICOTINIC ACID	RIBOFLAVIN
		%	%	%	%	*cal./gm.*	%	%	%	%	*μg./gm.*	*mg. %*	*μg./gm.*
A	White water bread, enriched	7.20	13.38	2.34	0.15	4.28	2.60	0.048	0.062	0.0051	4.2	3.3	0.7
B	White milk bread	7.33	14.75	1.92	0.14	4.34	2.90	0.121	0.166	0.0046	1.1	1.0	1.7
C	White milk bread, enriched	6.06	15.00	1.29	0.18	4.21	2.99	0.122	0.170	0.0084	3.8	2.8	1.6
D	Whole wheat water bread	6.85	15.75	4.62	2.70	4.38	3.04	0.064	0.383	0.0061	3.6	3.9	1.7
E	White water bread, enriched, plus Ca and riboflavin	4.18	14.06	3.26	0.43	4.24	2.62	0.126	0.179	0.0066	4.1	2.2	1.5
F	White milk bread, enriched	4.94	14.75	2.50	0.12	4.19	2.75	0.122	0.176	0.0080	3.5	2.4	1.7
G	Whole wheat water bread	9.04	14.88	4.00	2.74	4.16	2.84	0.061	0.398	0.0040	3.0	3.7	1.4 [1]
H	White milk bread	8.94	14.00	2.45	0.23	4.09	3.55	0.120	0.150	0.0015	0.87	1.3	1.3 [1]
J	Whole wheat milk bread	7.63	15.06	3.72	2.50	4.20	3.67	0.126	0.422	0.0042	3.1	3.9	2.1 [1]

[1] The riboflavin content of breads G, H and J was determined by the microbiological method of Snell and Strong ('39).

equal to 0.8 to 3.2 mg. per pound; this specification cannot be put into effect until the commercial supply of riboflavin permits. The enriched white breads, C and F, containing 6% dried skim milk, contained per pound on a 38% moisture basis, 24.6 mg. of iron, 1.1 mg. of thiamine, 7.8 mg. of nicotinic acid, 0.5 mg. of riboflavin and 366 mg. of calcium.

The losses of thiamine, originally present in the dough, during baking and subsequent drying of the bread were quite inconsiderable, in agreement with some previous work (Hoffman, Schweitzer and Dalby, '40; Hanning, '41; Fincke and Little, '41). In five individual tests, the thiamine content of dough, fresh bread and dried bread in micrograms per gram of dry matter averaged 3.84, 3.83 and 3.89, respectively. In five other tests on dough and dried bread, an average loss of 13% was indicated, although in only two of the tests was a considerable loss observed. However, when the crust and crumb of the breads were analyzed separately, the crust contained 27% less thiamine than the crumb on the dry basis, confirming the observations of Martin ('41). We have been unable to detect any loss of riboflavin either during the baking or the drying of the experimental breads.

Body growth, blood and bone composition. The average body weight gains of the rats in the first two tests are plotted against time on experimental feeding in figures 1 and 2. From figure 1 it is evident that bread A, enriched water bread, promoted a definitely poorer growth than either breads B or C, containing dried skim milk, and that the rates of growth secured on the latter two breads are indistinguishable. These conclusions are supported by a statistical analysis of the individual data with reference to total gains in weight made and final body length attained, using the method of Student ('08, '25). The results of ad libitum feeding, plotted on the right side of the figure, merely exaggerate the difference in growth-promoting power between bread A and the other breads, an exaggeration brought about by a much greater consumption of the better breads. In the controlled feeding

tests, the rat consuming bread A set the pace in food consumption in all trios.

No significant differences between breads B and C were evident with reference to the ash content of the bones of the rats consuming them, averaging 61.79 and 61.50% of the dry

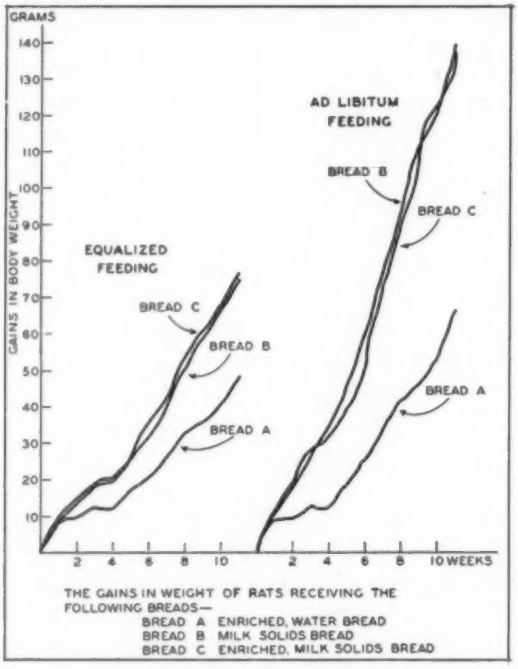

Figure 1

ether-extracted bones, respectively, in the controlled feeding test. However, in this test, the hemoglobin in the blood of the rats receiving bread C, enriched with iron, thiamine and nicotinic acid as well as with dried skim milk, was significantly ($P = 0.011$ [1]), if only slightly higher (14.62 gm. per 100 cc.)

[1] P = the probability that a given difference between two averages has been the result of a fortuitous combination of uncontrolled experimental conditions. If this probability is as small as 0.03 it may be disregarded, and the conclusion drawn that the difference observed was produced by the difference in the experimental conditions imposed.

than that for bread B (14.09 gm. per 100 cc.). The ad libitum feeding test failed to reveal significant differences between these two breads in bone or blood composition of the rats.

Breads B and C promoted significantly greater calcification of the bones than bread A, but no greater (or less) concentration of hemoglobin in the blood. Both controlled and ad libitum tests agree in these respects.

It is evident that the incorporation of non-fat milk solids in white bread to the extent of 6% of the flour, improves the growth-promoting and bone-forming values of the bread much more than does its enrichment with thiamine, iron and nicotinic acid. This superiority of non-fat milk solids is probably traceable to its content of calcium, phosphorus, riboflavin and superior protein. The addition of thiamine, nicotinic acid and iron to bread containing dried skim milk does not further improve its capacity of promoting growth, but it does, if only slightly, induce a greater concentration of hemoglobin in the blood, an effect that might have been more evident in these tests if the rats had been depleted of iron initially.

Inspection of figure 2 reveals a distinct inferiority in the growth-promoting capacity of bread E, enriched with calcium and riboflavin as well as with thiamine, nicotinic acid and iron, as compared with bread D, made with whole wheat flour, or as compared with bread F, containing skim milk powder as well as added thiamine, nicotinic acid and iron. This graphical observation is confirmed by statistical analysis of the individual gains in weight and body lengths attained, in both the controlled feeding test and the ad libitum feeding test. The observed differences are greatly exaggerated in the latter test due to greater acceptability of the D and F breads revealed both by a greater consumption of them when the intake of food was unrestricted, and by fewer food refusals [8] in the controlled feeding test. In the latter test, breads D and F are not to be distinguished statistically in the rate of growth

[8] The term "food refusal" refers to a leaving of some food from the preceding day's ration.

secured, but with ad libitum feeding more of bread D was consumed in ten of the eleven trios, although the growth rates induced were not significantly different.

A comparison of breads E and F, the latter containing dried skim milk in place of equivalent amounts of calcium and riboflavin in the former bread, with reference to bone and

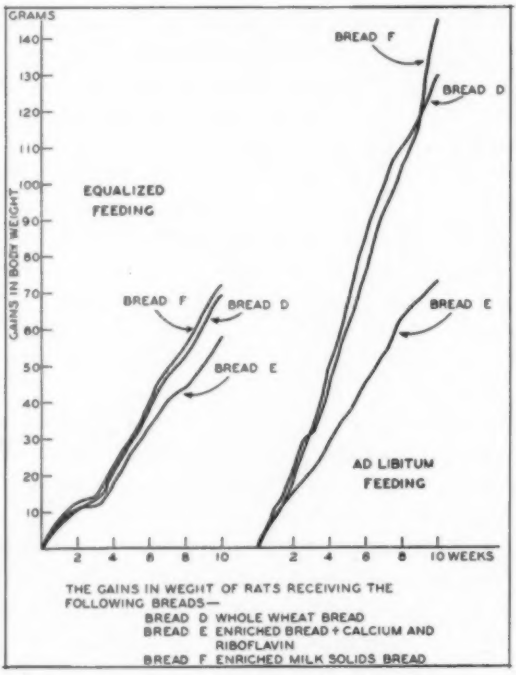

Figure 2

blood composition indicates a significantly greater hemoglobin concentration for the rats receiving the non-fat milk solids bread (14.32 as compared with 12.68 gm. per 100 cc.: $P = 0.008$), but a significantly smaller bone ash (59.51 as compared with 60.82%: $P = 0.0043$). These results were obtained with controlled feeding and were not revealed by ad libitum

feeding. The whole wheat bread, D, induced a significantly higher hemoglobin concentration of the blood than the fully enriched bread, E (14.10 vs. 12.68 gm. per 100 cc.: $P = 0.0006$), but a distinctly lower content of ash in the bones (56.66 vs. 60.82%, respectively: $P < 0.0001$). In this case, ad libitum feeding also showed similar differences between the two breads.

The whole wheat bread, D, which exhibited similar growth-promoting capacity to the enriched milk bread, F, also induced practically the same rate of hemoglobin production, but a distinctly lower calcification of the bones (56.70 vs. 59.76%, respectively: $P = 0.0004$). Ad libitum feeding supported the same conclusions.

The data secured on the breads of series 2 prove that enriched white bread containing 6% dried skim milk (F) is definitely superior in growth-promoting value and in hemoglobin production to enriched white bread to which calcium and riboflavin are added in amounts equivalent to 6% dried skim milk (E); however, the former is definitely, if only slightly, inferior to the latter in bone calcifying value although containing approximately the same contents of calcium and of phosphorus. Furthermore, enriched white bread with non-fat milk solids is the equal of whole wheat bread in the promotion of growth and in the production of blood hemoglobin, and superior to it in the promotion of bone calcification. Incidentally it is surprising how complete the bone calcification proved to be on diets containing only 0.12 to 0.13% calcium.

The breads of series 3 were tested on twelve trios of rats, each trio being fed its respective breads in amounts to promote approximately equal gains. The significant comparative results on growth, therefore, relate to intakes of food, and not to gains in body weight. The average food intakes of the rats fed breads G, H and J are plotted against time in figure 3. The differences in food intake among the three groups of rats are all significant statistically, as are also the differences in the numbers of food refusals plotted on the right side of the

figure. Evidently the growth-promoting value of white bread with non-fat milk solids (H) is greater than that of whole wheat bread (G), while a bread made from whole wheat flour with non-fat milk solids (J) is definitely better in the support of growth than either of the other two.

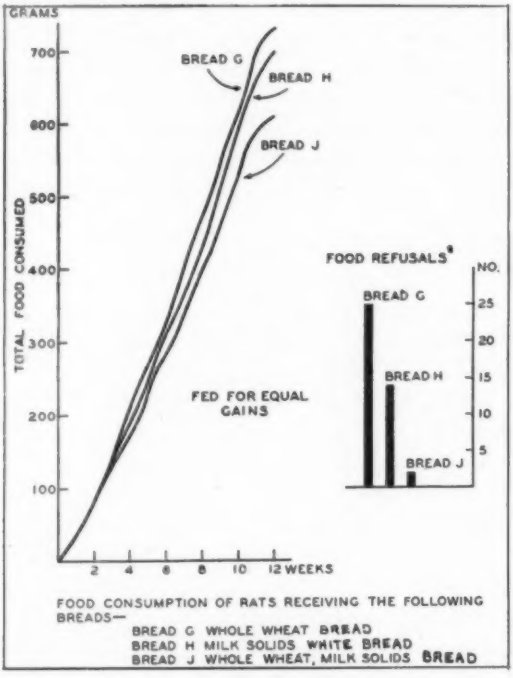

Figure 3

The average final hemoglobin concentrations of the blood were highest for the twelve rats consuming bread J (15.96 gm. per 100 cc.), intermediate for the twelve rats on bread G (15.28 gm. per 100 cc.) and lowest for the twelve rats on bread H (14.71 gm. per 100 cc.), but only the average difference between breads H and J is clearly significant ($P = 0.0003$); that between breads G and J was indicative ($P = 0.063$). In the ash content of the bones produced, the breads arranged

themselves in the following order: H (59.29%), J (57.67%) and G (53.73%). The average differences between all pairs of breads were highly significant.

Evidently, for purposes of growth, dried skim milk is a better supplement to white flour than the residues of the wheat berry discarded in its milling, though a combination of the two supplements is better than either alone. In this combination, the mill residues provide available iron in which skim milk is deficient, while skim milk provides available calcium in which the mill residues are deficient, and also improves the quality of the protein. The superior bone-forming value of bread H over bread J, in spite of similar contents of calcium, indicates the presence in whole wheat flour of constituents that impair calcium utilization, possibly the higher content of phosphorus or the nature of the compounds in which it is combined (McCance and Widdowson, '42).

The iron and vitamin contents of the experimental rats. Determinations of nicotinic acid, thiamine, riboflavin and iron were made upon some of the carcasses of the experimental rats in the tests on the breads of series 1 and 2, but not of series 3, with the average results summarized in table 2. These results have been expressed both as total contents and as concentrations, and differences between average results in the different sets of trios have been systematically analyzed statistically by the method of Student ('08, '25). The statements made below are supported by the statistical evidence thus secured.

Since rat-feeding tests cannot evaluate diets with respect to their contents of nicotinic acid, because of the ability of the rat to synthesize this vitamin (Shourie and Swaminathan, '40; Dann, '41; Huff and Perlzweig, '42), it was hoped, nevertheless, that a study of the nicotinic acid contents of the carcasses of the experimental rats would throw some light upon the biological availability of the dietary nicotinic acid. However, the average nicotinic acid concentrations of the various groups of rats varied within rather narrow limits,

TABLE 2

The iron and vitamin contents of the carcasses of some of the experimental rats.

NUTRIENT DETERMINED IN CARCASS	CONTROLLED FEEDING				AD LIBITUM FEEDING			
	Number of trios analyzed	Bread A	Bread B	Bread C	Number of trios analyzed	Bread A	Bread B	Bread C
Nicotinic acid per rat (mg.)	4	2.86	3.54 [1]	3.44	3	3.74 [2]	5.74	6.46
Nicotinic acid per gram of rat (μg.)	4	29.9	27.0 [1]	27.9	3	31.3 [2]	31.4	37.3
Thiamine per rat (μg.)	7	198	92	195	3	223	111	287
Thiamine per gram of rat (μg.)	7	2.13	0.75	1.60	3	2.02	0.64	1.74
Riboflavin per rat (μg.)	3	248	358	367	3	318	537	587
Riboflavin per gram of rat (μg.)	3	2.51	2.74	2.86	3	2.87	3.01	3.35
Iron per rat (mg.)	4	5.56	5.00	5.86	4	4.22	4.79	5.35
Iron per 100 gm. of rat (mg.)	4	5.77	3.96	4.51	4	5.15	3.14	3.42
		Bread D	Bread E	Bread F		Bread D	Bread E	Bread F
Nicotinic acid per rat (mg.)	4	4.31	3.43	4.16	4	5.10	3.38	5.30
Nicotinic acid per gram of rat (μg.)	4	35.7	32.0	33.9	4	32.7	31.7	33.7
Thiamine per rat (μg.)	8	216	200	208	5	322	245	328
Thiamine per gram of rat (μg.)	8	1.80	1.82	1.73	5	1.90	2.18	1.96
Riboflavin per rat (μg.)	3	359	291	331	3	571	427	635
Riboflavin per gram of rat (μg.)	3	3.38	2.98	3.11	3	3.26	3.72	3.31
Iron per rat (mg.)	4	5.10	4.37	4.80	4	5.65	3.80	5.48
Iron per 100 gm. of rat (mg.)	4	4.41	4.31	3.96	4	3.99	3.86	4.12

[1] Averages for only three rats.

[2] Averages for only two rats.

27.0 to 37.3 μg. per gram, irrespective of the dietary intake. In particular, the low nicotinic acid content of bread B, only 1.0 mg. per cent, was not associated with definitely low concentrations of this vitamin in the carcass. As a result of this lack of correlation between nicotinic acid intake and its concentration in the tissues, the total nicotinic acid contents of the rats were dependent mainly upon the total growth secured. As a consequence, where non-fat milk solids increased growth (breads B and C vs. bread A; bread F vs. bread E), they also increased the nicotinic acid present in the tissues, although not always significantly, possibly because of the small numbers of analyses secured. However, bread C, white milk bread enriched, induced a significantly greater amount of nicotinic acid in growing rats than did bread A, white bread, enriched.

In contrast to nicotinic acid, the thiamine concentrations of the rat carcasses were sensitive to low thiamine intakes, particularly the low thiamine content of bread B, a white bread containing dried skim milk. Even though this bread promoted a considerably more rapid growth than enriched bread A, the total thiamine content of the rats subsisting on bread B was less than half that of the rats subsisting on bread A. When skim milk solids are added to enriched bread (bread C vs. bread A), a significant reduction in the carcass concentration of thiamine occurred, from an average of 2.13 μg. per gram to 1.60 μg. per gram. This reduction may be explained, in part at least, by the smaller thiamine content of bread C, 3.8 μg. per gram, as compared with 4.2 μg. per gram for bread A. Whole wheat bread, D, maintained as high a thiamine concentration in the tissues and as high a total thiamine retention as enriched white bread containing skim milk solids, bread F.

The riboflavin concentrations of the tissues were not particularly responsive to the riboflavin intake. Thus, the low riboflavin content of bread A, enriched, did not induce a correspondingly low riboflavin concentration in the carcass, as compared, for example, with bread C, an enriched bread

with skim milk solids. The small difference, 2.51 and 2.86 μg. per gram, respectively, was, however, statistically significant. Hence, the effects of non-fat milk solids (breads B, C and F), or riboflavin (bread E), or whole wheat flour (bread D), upon riboflavin retention in the tissues were largely dependent upon the growth secured. As a matter of fact, no significant differences in riboflavin retention were demonstrated between breads B and C, or among breads D, E, and F. The retention of riboflavin in bread A, an enriched white bread, was definitely inferior to that in breads B or C.

No obvious relationship was observed between the intake of iron and the concentration of this element in the carcasses of the rats. It is true that bread B, a white skim milk bread, produced carcasses with concentrations of iron significantly less than did breads A or C both enriched with iron, but in the second series of breads, the lowest level of carcass iron was produced in rats receiving the bread with the highest iron content, bread F. Furthermore, significant differences in the iron concentration of the carcasses were not always associated with significant differences in the concentration of hemoglobin in the blood (i.e., bread A vs. bread B), and vice versa (i.e., bread D vs. bread E). However, all bread comparisons indicating a significant difference in hemoglobin concentration of the blood also indicated significant differences in the total iron content of the carcasses, i.e., bread B vs. bread C; bread D vs. bread E); in fact, these comparisons were the only ones showing significant differences in total carcass iron. Skim milk solids contributed practically no iron to the breads in which they were incorporated, and the addition of iron (bread C vs. bread B) was definitely reflected in the iron and hemoglobin analyses. Whole wheat bread, D, gave as good or better iron retention and hemoglobin production as breads enriched with iron salts, E and F.

CONCLUSIONS

The controlled and ad libitum feeding tests with growing rats upon nine experimental breads varying in their contents

of non-fat milk solids, "enriching" nutrients, and white or whole wheat flour, yielded results, together with the analyses of the breads themselves, that support the following conclusions:

1. The baking of bread by a commercial method leads to inconsiderable destruction of thiamine in the dough and to inappreciable destruction of riboflavin.

2. The ad libitum method of feeding, compared with methods involving well-considered control of the intake of food by comparative experimental animals, exaggerates the biological effect of large differences in the nutritive value of experimental diets, but may obscure small differences.

3. The incorporation of non-fat milk solids in white bread to the extent of 6% of the flour, improves the growth-promoting and bone calcifying values of the bread much more than does its enrichment with thiamine, nicotinic acid and iron. The "enrichment" of white bread containing skim milk solids does not further improve its capacity of promoting growth, but it does, if only slightly, induce a greater concentration of hemoglobin in the blood.

4. Enriched white bread containing 6% of dried skim milk is definitely superior in growth-promoting value and in hemoglobin production to enriched white bread supplemented to an equivalent extent with dicalcium phosphate and riboflavin; however, the former is definitely, if only slightly, inferior to the latter in bone calcifying value.

5. Enriched white bread with skim milk solids is the equal of whole wheat bread in the promotion of growth and in the production of blood hemoglobin and is distinctly superior to it in the promotion of bone calcification.

6. For purposes of growth, skim milk solids is a better supplement to white flour than the residues of the wheat berry discarded in its milling, though a combination of the two supplements is better than either alone.

7. There is something in whole wheat, as compared with patent white, flour that impairs calcium utilization.

8. Regardless of the intake of nicotinic acid, the amount present in the tissues of the growing rat depends upon the amount of growth induced.

9. The thiamine concentration of the tissues, much more than the concentration of riboflavin, is sensitive to variations in the intake of the corresponding vitamin.

10. Non-fat milk solids to the extent of 6% of the white flour may increase the riboflavin content of white bread to the level of that of whole wheat bread.

11. Where a change in bread formula induced an increase in the concentration of hemoglobin in blood it also caused a greater concentration of iron in the whole body, but the reverse was not always true.

12. Enrichment of white bread with thiamine, nicotinic acid and iron (and also with riboflavin) is not the equivalent of enrichment with non-fat milk solids. Non-fat milk solids definitely improves the nutritive value both of enriched white bread and of whole wheat bread.

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THE BODILY STORE OF VITAMIN A AS INFLUENCED BY AGE AND BY FOOD

ALICE B. ROHRER AND H. C. SHERMAN
Department of Chemistry, Columbia University, New York City

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Early experiments, in which the bodily stores of vitamin A were judged by the survival periods of standardized laboratory rats on otherwise good diets devoid of the vitamin, showed that such stores varied widely with the nutritional background of the animal, whether tested at the age of 2, 3, 4 or 6 months (Sherman and Cammack, '26). Among rats kept uniformly on a diet which contained 6 International Units (I.U.) of vitamin A per gram of air-dry food, the maximal ability to survive deprivation of this vitamin was reached at an age of 6 months (Sherman and Storms, '25). Sherman and Boynton ('25), who assayed the vitamin A contents of the different tissues by a feeding method, and Baumann, Riising and Steenbock ('34), who used a colorimetric method based on the antimony trichloride reaction, agreed that over nine-tenths of the bodily store of vitamin A in a well-nourished rat is carried in the liver; the former authors found a barely measurable amount in skeletal muscle while the latter reported their tests of muscle as "negative." More recently the quantitative study of the influence of food and of age upon bodily stores of vitamin A has been resumed with the use of new methods. In the experiments of Kao and Sherman ('40) and in those here reported the liver and muscle tissues of experimental animals have been assayed for vitamin A by the Sherman and Todhunter ('34) single-feeding method modified to include side-by-side comparisons of the material under investigation with the U. S. P. standard reference cod-liver oil.

EXPERIMENTAL

As the body stores its surplus of vitamin A so predominantly in the liver, attention is here directed chiefly to our findings upon liver tissue. However, the muscles of the same animals were also assayed because of the theoretical importance of the question whether the very low concentration of vitamin A in muscle tissue is appreciably influenced by the level of nutritional intake.

Three diets were here fed: (a) a mixture of one part dried whole milk with five parts ground whole wheat plus table salt (2% of the weight of the wheat), the air-dry food mixture having a value of 3 I.U. of vitamin A per gram; (b) the same plus sufficient cod-liver oil to bring the vitamin A value to 6 I.U. per gram; and (c) the same with further enrichment by cod-liver oil to 12 I.U. per gram. The animals had free

TABLE 1

Influence of level of intake upon vitamin A content of liver and muscle: Data in International Units per gram of fresh tissue.

AT AGE OF	LIVER			MUSCLE		
	At intake level of			At intake level of		
	3 I.U./gm. of diet	6 I.U./gm. of diet	12 I.U./gm. of diet	3 I.U./gm. of diet	6 I.U./gm. of diet	12 I.U./gm. of diet
30 days — mean	7.6	42.6	91.1	0.33	0.45	0.70
P.E.[1] of mean	± 1.9	± 4.0	± 6.3	± 0.12	± 0.13	± 0.13
No. of cases	14	17	17	18	18	17
60 days — mean	5.6	53.2	163.8	0.35	0.42	0.80
P.E.[1] of mean	± 1.3	± 6.5	± 10.6	± 0.11	± 0.10	± 0.18
No. of cases	18	18	19	20	20	20
Change from 30th to 60th day	— 2.0	10.6	72.7			
P.E.[1] of difference	± 2.3	± 7.6	± 11.9			
Difference from 1st level						
at 30 days		35 ± 4.5[1]	84 ± 6.6[1]			
at 60 days		48 ± 6.6	158 ± 10.1			

[1] This is the classical Probable Error.

access to their respective diets and to distilled water. The parents of the experimental animals had subsisted upon the respective diets since infancy.

Healthy, typical animals from these three nutritional backgrounds (and all of the same genetic strain) were killed at the fixed ages of 30 and 60 days, and their freshly dissected liver and muscle tissues were assayed with the results summarized in table 1.

DISCUSSION

Even at the age of 30 days (approximate "end of infancy" in the rat) the stores of vitamin A in the liver differ widely with the background of nutritional intake of this vitamin, as will be seen from table 1; and at 60 days of age these differences are still greater. At each age, the difference due to each increase in the level of intake (3 to 6 to 12 I.U. per gram of food) is statistically "undoubtedly significant." Statistically also there is undoubtedly an increase in the vitamin A content of the livers of the animals on the highest intake level during the second month of their lives; but no such change during the same age period is here established as statistically significant in the animals receiving either 3 or 6 I.U. of vitamin A per gram of dry food. In other words, the animals receiving 3 or 6 I.U. per gram of food showed practically no change in bodily concentration of vitamin A while making normal growth in bodily weight during the second month of their lives; while those receiving 12 I.U. per gram of food did increase their bodily concentration of this vitamin very significantly during the same age period.

Interpretation of the findings of our assays of the skeletal muscle tissue of the same animals (table 1) is more difficult because even at very liberal levels of feeding the animals did not store in their muscles much more than a barely measurable concentration of vitamin A. Probably, however, the present findings may be regarded as establishing a statistically significant — though in absolute terms very small — increase in the concentration-level of vitamin A in muscle tissue as a

result of liberal increase in the level of nutritional intake. This evidence may be coordinated with that of previous and parallel work as follows: Sherman and Boynton ('25) reported the presence of amounts too small to permit measurement of differences. Baumann, Riising and Steenbock ('34) found no vitamin A in muscle (or in blood). Kao and Sherman ('40) in a comparatively short series of cases, all being animals of the same season, found a barely measurable amount in the muscles of rats on low vitamin A intake, and a slightly higher, but still very low, concentration of this vitamin in the muscles when the level of nutritional intake was increased.

The present paper shows the data of a larger number of cases distributed over all seasons of 2 years. From these data (table 1) it may be seen that, while most of the differences are so small as to be of doubtful statistical significance, there is a perceptible trend to higher values in the muscles of the animals on the higher levels of intake. This is in agreement with the largely parallel but experimentally independent work reported by Kao and Sherman ('40).

Among the animals from families on any one level of intake, neither series shows any distinct difference in the vitamin A content of muscles at 30 and 60 days of age, respectively. This does not exclude the possibility of a slight difference — too small to measure by available methods — as would seem theoretically probable in view of the differences in blood plasma concentrations found by Lewis, Bodansky, Falk and McGuire ('42) in their studies of rats fed at much more widely different levels of vitamin A intake.

SUMMARY

Offspring of rat families whose diets, otherwise similar, contained respectively 3, 6, and 12 I.U. of vitamin A per gram of air-dry food, were killed at ages of 30 and 60 days and the vitamin A values of their liver and muscle tissues determined. Whether compared at the age of 30 or 60 days the vitamin A in the liver was found to have been decidedly influenced by the level of nutritional intake of this vitamin.

The skeletal muscles of the same animals showed differences in the same direction, but so small as to be of doubtful statistical significance. Storage of vitamin A in the body, as reflected by the concentration in the liver, was found to have continued in the second age period studied when the level of nutritional intake of the vitamin was high but not when it was near the minimal-adequate level.

The aid of grants from the Rockefeller Foundation is gratefully acknowledged.

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RÉSUMÉ OF THE COUNCIL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

In the absence of a regular meeting of the Institute, the Council met in special session in Detroit on March 29, 1943, at the Rackham Memorial Building of the University of Michigan.

Changes in officers were announced as follows: to be President (following Dr. Maynard's resignation), Dr. H. B. Lewis; to be Vice-President, Dr. Icie Macy Hoobler.

The following were elected to membership:

Otto A. Bessey — Grace A. Goldsmith
Henrik Dam — Harold P. Morris
David T. Smith

The dues for 1943–1944 were fixed at $2.00 per member and it was recommended that $1.00 per member be paid by the Treasurer in support of Federation Proceedings. The Council approved an allotment of $25.00 each to the Secretary and Treasurer for extra secretarial assistance.

With regard to the dues and subscription to The Journal of Nutrition of members who have entered military service, it was decided to bill these members as usual, and then, if requested, to suspend them from membership without prejudice, for the duration after which they will be reinstated if they so desire.

Announcement was made that the Mead Johnson & Company Award for 1943 had been granted to Dr. Vincent du Vigneaud and associates for their work on the Structure of Biotin. The Committee of Award was composed of A. D. Emmett, A. G. Hogan, E. W. McHenry, A. F. Morgan and R. M. Bethke, Chairman.

By recent vote, the membership approved the three amendments submitted for consideration; these amendments will be incorporated into the By-Laws of the Institute.

Because the annual meeting of the American Institute of Nutrition scheduled for the spring of 1943 was not held, there has been no formal meeting of the editorial board. The editor of The Journal of Nutrition submitted the following report:

During the year 1942 volumes 23 and 24 of The Journal of Nutrition were published; they contained 113 papers. There were submitted for publication during the year 170 papers; 41 were rejected, and 5 were withdrawn by the authors; the remaining papers are classified as "in suspense." The average number of articles printed per issue in volumes 23 and 24 proved to be 9.5, which approximates the 10 which editorial policy strives for.

During the year the Federal Government established a censorship of scientific publications. This has operated to delay somewhat the appearance of recent issues (since January, 1943). Another factor contributing to bring about such delay has been loss of trained personnel from the printing shop and related difficulties.

As was pointed out in the report submitted last year, solution of the problem of enlarging the Journal so as to secure more prompt publication of accepted articles depends almost entirely on securing additional income, in part through greater circulation and in part through acceptable advertising. Some progress has been made with respect to increase in the volume of advertising, but it is difficult to foresee how the war will affect this in the future.

Respectfully submitted,

ARTHUR H. SMITH, *Secretary*,
American Institute of Nutrition

INDEX

ABSORPTION and excretion of riboflavin, role played by gastrointestinal tract in 137
—— fat and vitamin A, in man, influence of lecithin feeding on.... 255
—— intestinal, of galactose in rat, rate of, effect of pantothenic acid on 403
Acid, ascorbic, content of peaches, influence of variety, size, and degree of ripeness upon 503
—— —— deficiency among Papago Indians 491
—— —— in twelve normal adults, renal threshold for, with a note on the state of tissue reserves of subjects on an intake of ascorbic acid approximating suggested daily allowance 185
—— —— nutrition of some college students 411
—— folic, and biotin, role of, in nutrition of rat 555
—— nicotinic, and riboflavin in rats, tissue content and balance of, effect of protein and B-vitamin levels of diet upon 173
—— —— content of common fruits and vegetables as prepared for human consumption 275
—— —— pantothenic acid, biotin and riboflavin content of several varieties of cheese. Value of dairy products in nutrition. III 463
—— —— storage in dog at different dose levels of the vitamin 341
—— pantothenic, effect of high levels of, on reproduction in rat and mouse 389
—— —— —— —— on rate of intestinal absorption of galactose in rat 403
—— —— nicotinic acid, riboflavin and biotin content of several varieties of cheese. Value of dairy products in nutrition. III 463
—— —— reversible inhibition of, by sulfapyridine. Effect of aromatic hydrocarbons on growth 471
Action, goitrogenic, of calcium and vitamin D 119
ADLERSBERG, DAVID, AND HARRY SOBOTKA. Influence of lecithin feeding on fat and vitamin A absorption in man 255
Age and food, bodily store of vitamin A as influenced by 605
Albino rat, critical temperature for, as affected by feeding 127
—— —— thiamine, riboflavin, pyridoxine and pantothenate deficiencies as affecting body composition of 7
ALLEN, DORIS I. The assay of vitamin B_1 in New Zealand materials by the thiochrome method 521
Animal and plant products, choline content of 441
—— —— vegetable fats, some, digestibility of 295
ANTHONY, E. KATHLEEN. See Sharpless and Anthony 239
—— See Sharpless, Sabol, Anthony and Argetsinger 119
ARGETSINGER, HELEN L. See Sharpless, Sabol, Anthony and Argetsinger 119
Aromatic hydrocarbons, effect of, on growth. Reversible inhibition of pantothenic acid by sulfapyridine 471
Arrangement, linear, of palatability of natural foods with an example of varietal preference in leguminosae and cruciferae by a new, rapid laboratory method 447
Ascorbic acid content of peaches, influence of variety, size, and degree of ripeness upon 503
—— —— deficiency among Papago Indians 491
—— —— in twelve normal adults, renal threshold for, with a note on the state of tissue reserves of subjects on an intake of ascorbic acid approximating suggested daily allowance 185
—— —— nutrition of some college students 411
Assay, biological, of pyridoxine (vitamin B_6) 571
—— of vitamin B_1 in New Zealand materials by the thiochrome method 521
Assays, thiamine, of foods using rat-growth method 395
Availability for human nutrition of vitamin C in raw cabbage and home-canned tomato juice 349
—— of calcium of some New Zealand vegetables 303
Award, Mead Johnson, for research on vitamin B complex for 1943.... 611

BALANCE and tissue content of riboflavin and nicotinic acid in rats, effect of protein and B-vitamin levels of diet upon 173
Basal metabolism of normal college women 245
BAUER, CLIFFORD D., AND CLARENCE P. BERG. Growth in mice fed diets rendered deficient in cystine, but not in methionine 497
BAVOUSETT, NEVA D. See McCrery, Lamb and Bavousett 245
BEADLES, J. R. See Mitchell, Hamilton and Shields 585
BERG, CLARENCE P. See Bauer and Berg 497
BERRYMAN, GEORGE H., AND CHARLOTTE CHATFIELD. A short method of calculating the nutritive value of diets 23
BETHKE, R. M. See Hunt, Burroughs, Bethke, Schalk and Gerlaugh ... 207
BEUK, JACK F. See Russell, Taylor and Beuk 275
Biological assay of pyridoxine (vitamin B_6) 571
—— determination of vitamin E 433
Biotin and folic acid, role of, in nutrition of rat 555
—— riboflavin, pantothenic acid and nicotinic acid content of several varieties of cheese. Value of dairy products in nutrition. III 463
BLACK, ALEX, AND RAYMOND W. SWIFT. The critical temperature for the albino rat as affected by feeding 127

Blood picture of rat, effects of inanition and riboflavin deficiency upon 511
BLOOM, EVELYN. See Sullivan, Bloom and Jarmol 463
BLOOMFIELD, ARTHUR L., AND WAY LEW. Increased resistance to ulcerative cecitis of rats on a diet deficient in the vitamin B complex 427
Bodily store of vitamin A as influenced by age and by food 605
Body composition of albino rat, thiamine, riboflavin, pyridoxine and pantothenate deficiencies as affecting 7
Bone calcification in growing rat, effect of dietary fat on 479
BORDEN, RUBY A. See Clayton and Borden 349
Bread and flour, enrichment of, utilization for hemoglobin regeneration of iron in salts used in 39
Bread, peeled wheat, vitamin B complex of 265
Breads, wheat, nutritive value of, contribution of non-fat milk solids to 585
Breast milk, factors affecting thiamine content of 49
BROWN, ALMEDA P., MARGARET L. FINCKE, JESSIE E. RICHARDSON, ELIZABETH N. TODHUNTER AND ELLA WOODS. Ascorbic acid nutrition of some college students 411
BUNKFELDT, RUDOLF, AND HARRY STEENBOCK. Vitellin, casein, liver and muscle tissue as sources of phosphorus for the rachitic rat.. 309
—— The effect of dietary fat on bone calcification in the growing rat.. 479
BURROUGHS, E. WISE. See Hunt, Burroughs, Bethke, Schalk and Gerlaugh 207
B-vitamin and protein levels of diet, effect of, upon tissue content and balance of riboflavin and nicotinic acid in rats 173

CABBAGE, raw, and home-canned tomato juice, availability for human nutrition of vitamin C in 349
Calcification, bone, in growing rat, effect of dietary fat on 479
Calcium and fat utilization, interrelation of 17
—— —— vitamin D, goitrogenic action of 119
—— of some New Zealand vegetables, availability of 303
Calves, young, utilization of urea by.. 197
Carotene content of tomatoes as influenced by various factors 539
Carotenoid metabolism, studies on. III. The effect of a high vitamin A diet on carotenoid metabolism of chickens 103
Casein, vitellin, liver and muscle tissue as sources of phosphorus for rachitic rat 309
Cattle, rumen content of, further studies on riboflavin and thiamine in. II. 207
Cecitis, ulcerative, of rats on diet deficient in vitamin B complex, increased resistance to 427
Cellulose and hemicellulose, disappearance of, from digestive tracts of children 59
CHATFIELD, CHARLOTTE. See Berryman and Chatfield 23
Cheese, several varieties of, riboflavin, pantothenic acid, nicotinic acid and botin content of. Value of dairy products in nutrition. III... 463
Chickens, carotenoid metabolism of, effect of high vitamin A diet on. Studies on carotenoid metabolism. III. 103
Children, digestive tracts of, disappearance of cellulose and hemicellulose from 59
—— normal, fat excretion by 379
Chloride, effect of, with high and low iodine supply on thyroid gland of rat 239
Choline and pyridoxine as factors in prevention of epithelial hyperplasia in forestomach of rats fed white flour 113
—— content of animal and plant products 441
—— —— —— feeds 1
Cirrhosis of liver, production of. Vitamin B complex studies in dogs 217
CLARKE, MIRIAM F., AND MARIE LECHYCKA. The biological assay of pyridoxine (vitamin B_6) 571
CLAYTON, MARY M., AND RUBY A. BORDEN. The availability for human nutrition of the vitamin C in raw cabbage and home-canned tomato juice 349
Committee, Mead Johnson award (American Institute of Nutrition) 611
Complex studies in dogs, vitamin B. Production of cirrhosis of liver.. 217
—— vitamin B, diet deficient in, increased resistance to ulcerative cecitis of rats on 427
—— —— ——, of peeled wheat bread 265
Composition, body, of albino rat, thiamine, riboflavin, pyridoxine and pantothenate deficiencies as affecting 7
Content, ascorbic acid, of peaches, influence of variety, size, and degree of ripeness upon 503
—— carotene, of tomatoes as influenced by various factors 539
—— choline, of animal and plant products 441
—— —— —— feeds 1
—— nicotinic acid, of common fruits and vegetables as prepared for human consumption 275
—— riboflavin, pantothenic acid, nicotinic acid and biotin, of several varieties of cheese. Value of dairy products in nutrition. III. 463
—— rumen, of cattle, further studies on riboflavin and thiamine in. II. 207
—— thiamine, of breast milk, factors affecting 49
—— tissue, and balance of riboflavin and nicotinic acid in rats, effect of protein and B-vitamin levels of diet upon 173
Contribution of non-fat milk solids to the nutritive value of wheat breads 585
Council meeting (American Institute of Nutrition) 611
Critical temperature for albino rat as affected by feeding 127
Cruciferae and leguminosae, varietal preference of, by a new, rapid laboratory method, on the linear arrangement of palatability of natural foods with an example of 447
Cucurbit seed globulins. II. Use as substitutes for edestin in experimental diets 99
CURRY, ROBERT. See Smith, Curry and Hawfield 341
CUSICK, PAUL L. See Williams, Mason, Cusick and Wilder 361
Cystine, diets rendered deficient in, but not in methionine, growth in mice fed 497

DAIRY products in nutrition, value of. III. Riboflavin, pantothenic acid, nicotinic acid and biotin content of several varieties of cheese 463
DAY, PAUL L. See Shukers and Day.. 511

Deficiencies, thiamine, rbioflavin, pyridoxine and pantothenate, as affecting body composition of albino rat 7
Deficiency, ascorbic acid, among Papago Indians 491
—— induced riobflavin, and riboflavin requirement of man, observations on 361
—— riboflavin, and inanition, effects of, upon blood picture of rat 511
Dermatoses, nutritional, in rat. VIII. Vitamin A deficiency 319
Determination, biological, of vitamin E 433
DEUEL, HARRY J., Jr. See Mattson and Deuel 103
Diet(s) deficient in vitamin B complex, increased resistance to ulcerative cecitis of rats on 427
—— experimental, edestin in, cucurbit seed globulins used as substitutes for 99
—— high vitamin A, effect of, on carotenoid metabolism of chickens. Studies on carotenoid metabolism. III. 103
—— highly purified, reproduction and lactation of mice on 161
—— rendered deficient in cystine, but not in methionine, growth in mice fed 497
—— short method of calculating nutritive value of 23
—— thiamine-low, of a type consumed by human beings, results of feeding rats 229
Dietary fat, effect of, on bone calcification in growing rat 479
Digestibility of some animal and vegetable fats 295
Disappearance of cellulose and hemicellulose from digestive tracts of children 59
Dog, nicotinic acid storage in, at different dose levels of the vitamin. 341
Dogs, vitamin B complex studies in. Production of cirrhosis of liver. 217
DORFMAN, FRIEDA. See Foster, Jones, Dorfman and Kobler 161
DOVE, W. FRANKLIN. On the linear arrangement of palatability of natural foods with an example of varietal preference in Leguminosae and Cruciferae by a new, rapid laboratory method 447

EDESTIN in experimental diets, cucurbit seed globulins used as substitutes for 99
Editor of The Journal of Nutrition, report of 612
Effect(s) of aromatic hydrocarbons on growth. Reversible inhibition of pantothenic acid by sulfapyridine 471
—— —— chloride with high and low iodine supply on the thyroid gland of the rat 239
—— —— dietary fat on bone calcification in growing rat 479
—— —— high levels of pantothenic acid on reproduction in rat and mouse 389
—— —— —— vitamin A diet on carotenoid metabolism of chickens. Studies on carotenoid metabolism. III. 103
—— —— inanition and riboflavin deficiency upon blood picture of rat 511
—— —— pantothenic acid on rate of intestinal absorption of galactose in rat 403
—— —— protein and B-vitamin levels of diet upon tissue content and balance of riboflavin and nicotinic acid in rats 173
ELKIN, C. A. See Pijoan, Elkin and Eslinger 491
ELLIOTT, RALPH F. See French and Elliott 17
ELLIS, GORDON H., AND KARL C. HAMMER. The carotene content of tomatoes as influenced by various factors 539
ELLIS, LILLIAN N., ANNA ZMACHINSKY AND HENRY C. SHERMAN. Experiments upon the significance of liberal levels of intake of riboflavin 153
ELLIS, L. N. See Ellis, Zmachinsky and Sherman 153
ELVEHJEM, C. A. See McIntire, Schweigert, Henderson and Elvehjem 143
ENDICOTT, ELIZABETH N. See Williams, Endicott, Shepherd, Galbraith and Macy 379
ENGEL, RUBEN W. The choline content of animal and plant products. 441
Enrichment of flour and bread, utilization for hemoglobin regeneration of iron in salts used in 39
Epithelial hyperplasia in forestomach of rats fed white flour, choline and pyridoxine as factors in prevention of 113
ESLINGER, C. O. See Pijoan, Elkin and Eslinger 491
EVANS, ROBERT J. See Rhian, Evans and St. John 1
EVANS, VIRGINIA J. See Sullivan and and Evans 319
Excretion and absorption of riboflavin, role played by gastrointestinal tract in 137
—— fat, by normal children 379
Experimental diets, edestin in, cucurbit seed globulins used as substitutes for 99
Experiments upon significance of liberal levels of intake of riboflavin 153

FACTORS affecting thiamine content of breast milk 49
—— in prevention of epithelial hyperplasia in forestomach of rats fed white flour, choline and pyridoxine as 113
—— various, carotene content of tomatoes as influenced by 539
Fat and calcium utilization, interrelation of 17
—— —— vitamin A absorption in man, influence of lecithin feeding on 255
—— dietary, effect of, on bone calcification in growing rat 479
—— excretion by normal children 379
—— metabolism and goiter 203
Fats animal and vegetable, some, digestibility of 295
Feeds, choline content of 1
FINCKE, MARGARET L. See Brown, Fincke, Richardson, Todhunter and Woods 411
Flour and bread, enrichment of, utilization for hemoglobin regeneration of iron in salts used in 39
—— white, choline and pyridoxine as factors in prevention of epithelial hyperplasia in forestomach of rats fed 113
Folic acid and biotin, role of, in nutrition of rat 555
Food and age, bodily store of vitamin A as influenced by 605
—— mixtures, certain low-cost, nutritive adequacy of 285
Foods, natural, on the linear arrangement of palatability of, with an example of varietal preference in leguminosae and cruciferae by a new, rapid laboratory method 447
Forestomach of rats fed white flour, choline and pyridoxine as factors in prevention of epithelial hyperplasia in 113

FOSTER, CLAIRE, JAMES H. JONES, FREIDA DORFMAN AND RUTH S. KOBLER. Reproduction and lactation of mice on highly purified diets 161
FOUTS, PAUL J. Vitamin B complex studies in dogs. Production of cirrhosis of liver 217
FREE, ALFRED H. See Leonards and Free 403
FRENCH, CYRUS E., AND RALPH F. ELLIOTT. The interrelation of calcium and fat utilization 17
Fruits and vegetables, common, nicotinic acid content of, as prepared for human consumption 275

GALACTOSE in rat, effect of pantothenic acid on rate of intestinal absorption of 403
GALBRAITH, HARRY. See Williams, Endicott, Shepherd, Galbraith and Macy 379
Gastrointestinal tract, role played by, in absorption and excretion of riboflavin 137
GERLAUGH, PAUL. See Hunt, Burroughs, Bethke, Schalk and Gerlaugh 207
Gland, thyroid, of rat, effect of chloride with high and low iodine supply on 239
Globulins, cucurbit seed. II. Use as substitutes for edestin in experimental diets 99
Goiter and fat metabolism 203
Goitrogenic action of calcium and vitamin D 119
GOTTLIEB, HOWARD L., FORREST W. QUACKENBUSH AND HARRY STEENBOCK. The biological determination of vitamin E 433
Growth, effect of aromatic hydrocarbons on. Reversible inhibition of pantothenic acid by sulfapyridine 471
—— in mice fed diets rendered deficient in cystine, but not in methionine 497

HAMILTON, T. S. See Mitchell, Hamilton and Shields 585
HAMNER, K. C. See Ellis and Hamner 539
HARRIS, PHILIP L. See Remington and Harris 203
HAUCK, HAZEL M. See Lewis, Storvick and Hauck 185
HAWFIELD, HAROLD. See Smith, Curry and Hawfield 341
HAWTHORNE, BETTY. See Lewis, Storvick and Hauck 185
Hemicellulose and cellulose, disappearance of, from digestive tracts of children 59
Hemoglobin regeneration of iron in salts used in enrichment of flour and bread, utilization for 39
HEMPHILL, FRANCES I., RUTH A. KOENIG AND JET C. WINTERS. Nutritive adequacy of certain low-cost food mixtures 285
HENDERSON, L. M. See McIntire, Schweigert, Henderson and Elvehjem 143
HIGANO, SHIZUKO. See Lewis, Storvick and Hauck 185
HIGGINS, GEORGE M., RAY D. WILLIAMS AND HAROLD L. MASON. Results of feeding rats a thiamine-low diet of a type consumed by human beings 229
HOAGLAND, RALPH, AND GEORGE G. SNIDER. Digestibility of some animal and vegetable fats 295
HOLMES, ARTHUR D. See Schroder, Satterfield and Holmes 503
HUBBELL, REBECCA B., HUBERT B. VICKERY AND LAURENCE S. NOLAN. Cucurbit seed globulins. II. Use as substitutes for edestin in experimental diets 99
HUMMEL, FRANCES C., MARION L. SHEPHERD AND ICIE G. MACY. Disappearance of cellulose and hemicellulose from the digestive tracts of children 59
HUNT, CHARLES H., EDWARD W. BURROUGHS, ROLAND M. BETHKE, ARTHUR F. SCHALK AND PAUL GERLAUGH. Further studies on riboflavin and thiamine in the rumen content of cattle. II. 207
Hydrocarbons, aromatic, effect of, on growth. Reversible inhibition of pantothenic acid by sulfapyridine 471
Hyperplasia, epithelial, in forestomach of rats fed white flour, choline and pyridoxine as factors in prevention of 113

INANITION and riboflavin deficiency, effects of, upon blood picture of rat 511
Indians, Papago, ascorbic acid deficiency among 491
Influence of lecithin feeding on fat and vitamin A absorption in man..., 255
—— —— variety, size, and degree of ripeness upon ascorbic acid content of peaches 503
Inhibition, reversible, of pantothenic acid by sulfapyridine. Effect of aromatic hydrocarbons on growth 471
Interrelation of calcium and fat utilization 17
Intestinal absorption of galactose in rat, rate of, effect of pantothenic acid on 403
Iodine supply, high and low, effect of chloride with, on thyroid gland of rat 239
Iron in salts, utilization for hemoglobin regeneration of, used in enrichment of flour and bread 39

JARMOL, JOAN. See Sullivan, Bloom and Jarmol 463
JEFFERSON, NELSON C. See West, Jefferson and Rivera 471
JONES, JAMES H. See Foster, Jones, Dorfman and Kobler 161
Juice, tomato, home-canned, and raw cabbage, availability for human nutrition of vitamin C in 349

KELLY, JOAN. The availability of the calcium of some New Zealand vegetables 303
KLEIGER, SARAH C. See Knott, Kleiger and Torres-Bracamonte 49
KNOTT, ELIZABETH M., SARAH C. KLEIGER AND FRANCISCO TORRES-BRACAMONTE. Factors affecting the thiamine content of breast milk 49
KOHLER, RUTH S. See Foster, Jones, Dorfman and Kobler 161
KOENIG, RUTH ADELE. See Hemphill, Koenig and Winters 285

LACTATION and reproduction of mice on highly purified diets.... 161
LAMB, MINA W. See McCrery, Lamb and Bavousett 245
LECHYCKA, MARIE. See Clarke and Lechycka 571
Lecithin feeding, influence of, on fat and vitamin A absorption in man 255

Leguminosae and cruciferae, varietal preference in, by a new, rapid laboratory method, on the linear arrangement of palatability of natural foods with an example of 447
LEONARDS, JACK R., AND ALFRED H. FREE. The effect of pantothenic acid on the rate of intestinal absorption of galactose in the rat .. 403
Levels, different dose, of vitamin, nicotinic acid storage in dog at 341
—— high, of pantothenic acid, effect of, on reproduction in rat and mouse 389
—— liberal, of intake of riboflavin, experiments upon significance of. 153
—— of diet, protein and B-vitamin, effect of, upon tissue content and balance of riboflavin and nicotinic acid in rats 173
LEW, WILLIAM. See Bloomfield and Lew 427
LEWIS, JANE S., CLARA A. STORVICK AND HAZEL M. HAUCK, with the cooperation of Isabel Patterson, Shizuko Higano and Betty Hawthorne. Renal threshold for ascorbic acid in twelve normal adults with a note on the state of tissue reserves of subjects on an intake of ascorbic acid approximating the suggested daily allowance 185
Linear arrangement of palatability of natural foods with an example of varietal preference in leguminosae and cruciferae by a new, rapid laboratory method 447
Liver, production of cirrhosis of. Vitamin B complex studies in dogs.. 217
—— vitellin, casein and muscle tissue as sources of phosphorus for rachitic rat 309
LIVERMORE, ARTHUR H. See Sealock and Livermore 265
LOOSLI, JOHN K., AND CLIVE M. MCCAY. Utilization of urea by young calves 197

MCCAY, C. M. See Loosli and McCay 197
MCCREERY, JONNIE, MINA W. LAMB AND NEVA D. BAVOUSETT. The basal metabolism of normal college women 245
MCINTIRE, JUNIUS M., BERNARD S. SCHWEIGERT, LAVELL M. HENDERSON AND CONRAD A. ELVEHJEM. The retention of vitamins in meat during cooking 143
MACY, ICIE G. See Hummel, Shepherd and Macy 59
—— See Williams, Endicott, Shepherd, Galbraith and Macy 379
Man, fat and vitamin A absorption in, influence of lecithin feeding on.. 255
—— observations on induced riboflavin deficiency and riboflavin requirement of 361
—— thiamine of, minimum daily rement of 71
MASON, HAROLD L. See Higgins, Williams and Mason 229
—— See Williams, Mason, Cusick and Wilder 361
—— See Williams, Mason and Wilder 71
MATTSON, FRED H., AND HARRY J. DEUEL, JR. Studies on carotenoid metabolism. III. The effect of a high vitamin A diet on the carotenoid metabolism of chickens... 103
Mead Johnson Award for research on vitamin B complex, report for the committee on 611
Meat, retention of vitamins in, during cooking 143
Metabolism, basal, of normal college women 245
Metabolism, carotenoid, studies on. III. The effect of a high vitamin A diet on carotenoid metabolism of chickens 103
—— fat, and goiter 203
Methionine, diets rendered deficient in cystine, but not in, growth in mice fed 497
Method, laboratory, new, rapid, on the linear arrangement of palatability of natural foods with an example of varietal preference in leguminosae and cruciferae by 447
—— rat-growth, thiamine assays of foods using 395
—— thiochrome, assay of vitamin B_1 in New Zealand materials by.... 521
Mice fed diets rendered deficient in cystine, but not in methionine, growth in 497
—— reproduction and lactation of, on highly purified diets 161
Milk, breast, factors affecting thiamine content of 49
—— solids, non-fat, contribution of, to nutritive value of wheat breads 585
MILLER, CAREY D. Thiamine assays of foods using the rat-growth method 395
Minimum daily requirement of thiamine of man 71
MITCHELL, HAROLD H., TOM S. HAMILTON AND JOHN B. SHIELDS, with technical assistance of J. R. Beadles. The contribution of nonfat milk solids to the nutritive value of wheat breads 585
MITCHELL, H. H. See Nakamura and Mitchell 39
MOORE, HOWARD P. See Voris and Moore 7
Mouse and rat, reproduction in, effect of high levels of pantothenic acid on 389
Muscle, vitellin, casein and liver tissue as sources of phosphorus for rachitic rat 309

NAKAMURA, FRANK I., AND HAROLD H. MITCHELL. The utilization for hemoglobin regeneration of the iron in salts used in the enrichment of flour and bread 39
Natural foods, on the linear arrangement of palatability of, with an example of varietal preference in leguminosae and cruciferae by a new, rapid laboratory method ... 447
New members elected (American Institute of Nutrition) 611
New Zealand materials, assay of vitamin B_1 in, by thiochrome method 521
—— vegetables, some, availability of calcium of 303
Nicotinic acid and riboflavin in rats, tissue content and balance of, effect of protein and B-vitamin levels of diet upon 173
—— —— content of common fruits and vegetables as prepared for human consumption 275
—— —— pantothenic acid, biotin and riboflavin content of several varieties of cheese. Value of dairy products in nutrition. III. 463
—— —— storage in the dog at different dose levels of the vitamin .. 341
NOLAN, LAURENCE S. See Hubbell, Vickery and Nolan 99
Nutrition, ascorbic acid, of some college students 411
—— —— human, of vitamin C in raw cabbage and home-canned tomato juice, availability for 349
—— of rat, role of folic acid and biotin in 555
—— value of dairy products in. III. Riboflavin, pantothenic acid, nicotinic acid and biotin content of several varieties of cheese 463

Nutritional dermatoses in the rat. VIII. Vitamin A deficiency 319
Nutritive adequacy of certain low-cost food mixtures 285
—— value of diets, short method of calculating 23
—— —— —— wheat breads, contribution of non-fat milk solids to 585

OBSERVATIONS on induced riboflavin deficiency and riboflavin requirement of man 361
Officers for 1943–44 (American Institute of Nutrition) 611

PALATABILITY of natural foods, on the linear arrangement of, with an example of varietal preference in leguminosae and cruciferae by a new, rapid laboratory method 447
Pantothenate, thiamine, riboflavin and pyridoxine deficiencies as affecting body composition of albino rat 7
Pantothenic acid, effect of high levels of, on reproduction in rat and mouse 389
—— —— —— —— on rate of intestinal absorption of galactose in rat 403
—— —— nicotinic acid, riboflavin and biotin content of several varieties of cheese. Value of dairy products in nutrition. III. 463
—— —— reversible inhibition of, by sulfapyridine. Effect of aromatic hydrocarbons on growth 471
Papago Indians, ascorbic acid deficiency among 491
PATTERSON, ISABEL. See Lewis, Storvick and Hauck 185
Peaches, influence of variety, size, and degree of ripeness upon ascorbic acid content of 503
Peeled wheat bread, vitamin B complex of 265
PENNINGTON, DOROTHY. See Taylor, Pennington and Thacker 389
PERLZWEIG, WILLIAM A. See Sarett and Perlzweig 173
Phosphorus, sources of, for rachitic rat, vitellin, casein, liver and muscle tissue as 309
PIJOAN, MICHEL, CARRIE A. ELKIN AND CHESTER O. ESLINGER. Ascorbic acid deficiency among Papago Indians 491
Plant and animal products, choline content of 441
Proceedings of the American Institute of Nutrition, tenth annual meeting (special session) 611
Protein and B-vitamin levels of diet, effect of, upon tissue content and balance of riboflavin and nicotinic acid in rats 173
Purified diets, highly, reproduction and lactation of mice on 161
Pyridoxine and choline as factors in prevention of epithelial hyperplasia in forestomach of rats fed white flour 113
—— biological assay of (vitamin B_6) 571
—— thiamine, riboflavin and pantothenate deficiencies as affecting body composition of albino rat ... 7

QUACKENBUSH, FORREST W. See Gottlieb, Quackenbush and Steenbock 433

RACHITIC rat, vitellin, casein, liver and muscle tissue as sources of phosphorus for 309
Rat(s) albino, critical temperature for, as affected by feeding 127
—— —— thiamine, riboflavin, pyridoxine and pantothenate deficiencies as affecting body composition of 7
—— and mouse, reproduction in, effect of high levels of pantothenic acid on 389
—— blood picture of, effects of inanition and riboflavin deficiency upon 511
—— fed white flour, forestomach of, choline and pyridoxine as factors in prevention of epithelial hyperplasia in 113
—— galactose in, effect of pantothenic acid on rate of intestinal absorption of 403
—— growing, effect of dietary fat on bone calcification in 479
—— growth method, thiamine assays of foods using 395
—— nutrition of, role of folic acid and biotin in 555
—— nutritional dermatoses in. VIII. Vitamin A deficiency 319
—— on diet deficient in vitamin B complex, increased resistance to ulcerative cecitis of 427
—— rachitic, vitellin, casein, liver and muscle tissue as sources of phosphorus for 309
—— results of feeding, a thiamine-low diet of a type consumed by human beings 229
—— thyroid gland of, effect of chloride with high and low iodine supply on 239
—— tissue content and balance of riboflavin and nicotinic acid in, effect of protein and B-vitamin levels of diet upon 173
Rate of intestinal absorption of galactose in rat, effect of pantothenic acid on 403
Regeneration, hemoglobin, of iron in salts used in enrichment of flour and bread, utilization for 39
REMINGTON, ROE E., AND PHILIP L. HARRIS. Fat metabolism and goiter 203
Renal threshold for ascorbic acid in twelve normal adults with a note on the state of tissue reserves of subjects on an intake of ascorbic acid approximating suggested daily allowance 185
Reproduction and lactation of mice on highly purified diets 161
—— in rat and mouse, effect of high levels of pantothenic acid on.... 389
Requirement, minimum daily, of thiamine of man 71
—— riboflavin, and induced riboflavin deficiency of man, observations on 361
Resistance, increased, to ulcerative cecitis of rats on a diet deficient in vitamin B complex 427
Results of feeding rats a thiamine-low diet of a type consumed by human beings 229
Retention of vitamins in meat during cooking 143
RHIAN, MORRIS, ROBERT J. EVANS AND JAMES L. ST. JOHN. The choline content of feeds 1
Riboflavin, absorption and excretion of, role played by gastrointestinal tract in 137
—— and nicotinic acid in rats, tissue content and balance of, effect of protein and B-vitamin levels of diet upon 173
—— —— thiamine in rumen content of cattle, further studies on. II. 207
—— deficiency and inanition, effects of, upon blood picture of rat.... 511
—— —— induced, and riboflavin requirement of man, observations on 361

Riboflavin, experiments upon significance of liberal levels of intake of 153
—— pantothenic acid, nicotinic acid and biotin content of several varieties of cheese. Value of dairy products in nutrition. III. 463
—— thiamine, pyridoxine and pantothenate deficiencies as affecting body composition of albino rat 7
Richardson, Jessie E. See Brown, Fincke, Richardson, Todhunter and Woods 411
Rivera, Raven E. See West, Jefferson and Rivera 471
Rohrer, Alice B., and Henry C. Sherman. The bodily store of vitamin A as influenced by age and by food 605
Role of folic acid and biotin in nutrition of rat 555
—— played by gastrointestinal tract in absorption and excretion of riboflavin 137
Rumen content of cattle, further studies on riboflavin and thiamine in. II. 207
Russell, Walter C., Milton W. Taylor and Jack F. Beuk. The nicotinic acid content of common fruits and vegetables as prepared for human consumption 275

Sabol, Marie. See Sharpless and Sabol 113
—— See Sharpless, Sabol, Anthony and Argetsinger 119
Salts, iron in, utilization for hemoglobin regeneration of, used in enrichment of flour and bread 39
Sarett, Herbert P., and William A. Perlzweig. The effect of protein and B-vitamin levels of the diet upon the tissue content and balance of riboflavin and nicotinic acid in rats 173
Satterfield, G. H. See Schroder, Satterfield and Holmes 503
Schalk, A. F. See Hunt, Burroughs, Bethke, Schalk and Gerlaugh 207
Schroeder, George M., George H. Satterfield and Arthur D. Holmes. The influence of variety, size and degree of ripeness upon the ascorbic acid content of peaches 503
Schweigert, B. S. See McIntire, Schweigert, Henderson and Elvehjem 143
Sealock, Robert R., and Arthur H. Livermore. The vitamin B complex of peeled wheat bread 265
Seed globulins, cucurbit. II. Use as substitutes for edestin in experimental diets 99
Selye, Hans. The role played by the gastrointestinal tract in the absorption and excretion of riboflavin 137
Sharpless, George R., and Erin K. Anthony. The effect of chloride with high and low iodine supply on the thyroid gland of the rat 239
Sharpless, George R., and Marie Sabol. Choline and pyridoxine as factors in prevention of epithelial hyperplasia in the forestomach of rats fed white flour 113
Sharpless, George R., Marie Sabol, Erin K. Anthony and Helen L. Argetsinger. Goitrogenic action of calcium and vitamin D 119
Shepherd, Marion L. See Hummel, Shepherd and Macy 59
—— See Williams, Endicott, Shepherd, Galbraith and Macy 379
Sherman, H. C. See Ellis, Zmachinsky and Sherman 153
—— See Rohrer and Sherman 605
Shields, J. B. See Mitchell, Hamilton and Shields 585
Short method of calculating nutritive value of diets 23
Shukers, Carroll F., and Paul L. Day. The effects of inanition and riboflavin deficiency upon the blood picture of the rat 511
Smith, Susan G., Robert W. Currey and Harold H. Hawfield. Nicotinic acid storage in the dog at different dose levels of the vitamin 341
Snider, George G. See Hoagland and Snider 295
Sobotka, Harry. See Adlersberg and and Sobotka 255
Sprague, K. L. See Welch and Wright 555
St. John, J. L. See Rhian, Evans and St. John 1
Steenbock, Harry. See Bunkfeldt and Steenbock 309, 479
—— See Gottlieb, Quackenbush and Steenbock 433
Storage, nicotinic acid, in dog at different dose levels of the vitamin 341
Store, bodily, of vitamin A as influenced by age and by food 605
Storvick, Clara A. See Lewis, Storvick and Hauck 185
Studies, further, on riboflavin and thiamine in the rumen content of cattle. II. 207
—— on carotenoid metabolism. III. The effect of a high vitamin A diet on the carotenoid metabolism of chickens 103
Sulfapyridine, reversible inhibition of pantothenic acid by. Effect of aromatic hydrocarbons on orgwth 471
Sullivan, Maurice, and Virginia J. Evans. Nutritional dermatoses in the rat. VIII. Vitamin A deficiency 319
Sullivan, Royal A., Evelyn Bloom and Josephine J. Jarmol. The value of dietary products in nutrition. III. The riboflavin, pantothenic acid, nicotinic acid and biotin content of several varieties of cheese 463
Swift, Raymond W. See Black and Swift 127

Taylor, Alfred, Dorothy T. Pennington and Juanita I. Thacker. The effect of high levels of pantothenic acid on reproduction in the rat and the mouse 389
Taylor, M. Wight. See Russell, Taylor and Beuk 275
Temperature, critical, for albino rat as affected by feeding 127
Thacker, Juanita. See Taylor, Pennington and Thacker 389
Thiamine and riboflavin in rumen content of cattle, further studies on. II. 207
—— assays of foods using the rat-growth method 395
—— content of breast milk, factors affecting 49
—— low diet of a type consumed by human beings, results of feeding rats 229
—— of man, minimum daily requirement of 71
—— riboflavin, pyridoxine and pantothenate deficiencies as affecting body composition of albino rat 7
Thiochrome method, assay of vitamin B_1 in New Zealand materials by 521
Threshold, renal, for ascorbic acid in twelve normal adults with a note on the state of tissue reserves of subjects on an intake of ascorbic acid approximating suggested daily allowance 185

Thyroid gland of rat, effect of chloride with high and low iodine supply on 239
Tissue content and balance of riboflavin and nicotinic acid in rats, effect of protein and B-vitamin levels of diet upon 173
—— reserves of subjects on an intake of ascorbic acid approximating suggested daily allowance, renal threshold for ascorbc acid in twelve normal adults with a note on 185
—— vitellin, casein, liver and muscle, as sources of phosphorus for rachitic rat 309
TODHUNTER, E. NEIGE. See Brown, Fincke, Richardson, Todhunter and Woods 411
Tomato juice, home-canned, and raw cabbage, availability for human nutrition of vitamin C in 349
Tomatoes, carotene content of, as influenced by various factors 539
TORRES-BRACAMONTE, F. See Knott, Kleiger and Torres-Bracamonte.. 49
Tract, gastrointestinal, role played by, in absorption and excretion of riboflavin 137
Tracts, digestive, of children, disappearance of cellulose and hemicellulose from 59

ULCERATIVE cecitis of rats on diet deficient in vitamin B complex, increased resistance to.... 427
Urea, utilization of, by young calves.. 197
Utilization, calcium and fat, interrelation of 17
—— for hemoglobin regeneration of iron in salts used in enrichment of flour and bread 39
—— of urea by young calves 197

VALUE, nutritive, of diets, short method of calculating 23
—— —— —— wheat breads, contribution of non-fat milk solids to 585
—— of dairy products in nutrition. III. Riboflavin, pantothenic acid, nicotinic acid and biotin content of several varieties of cheese.... 463
Vegetable and animal fats, some, digestibility of 295
Vegetables and fruits, common, nicotinic acid content of, as prepared for human consumption 275
—— New Zealand, some, availability of calcium of 303
VICKERY, HUBERT B. See Hubbell, Vickery and Nolan 99
Vitamin, nicotinic acid storage in dog at different dose levels of 341
Vitamins in meat during cooking, retention of 143
Vitamin A and fat absorption in man, influence of lecithin feeding on.. 255
—— —— bodily store of, as influenced by age and by food..... 605
—— —— deficiency, Nutritional dermatoses in rat. VIII. 319
—— —— diet, high, effect of, on carotenoid metabolism of chickens. Studies on carotenoid metabolism. III. 103
Vitamin B and protein levels of diet, effect of, upon tissue content and balance of riboflavin and nicotinic acid in rats 173
—— —— complex, diet deficient in, increased resistance to ulcerative cecitis of rats on 427
—— —— —— of peeled wheat bread 265
—— —— —— studies in dogs. Production of cirrhosis of liver.... 217
—— B_{12}, assay of, in New Zealand materials by the thiochrome method 521
—— B_6 (pyridoxine), biological assay of 571
—— C in raw cabbage and home-canned tomato juice, availability for human nutrition of 349
—— D and calcium, goitrogenic action of 119
—— E, biological determination of.. 433
Vitellin, casein, liver and muscle tissue as sources of phosphorus for rachitic rat 309
VORIS, LEROY, AND HOWARD P. MOORE. Thiamine, riboflavin, pyridoxine and pantothenate deficiencies as affecting the body composition of the albino rat 7

WELCH, A. D., AND LEMUEL D. WRIGHT. The role of "folic acid" and biotin in the nutrition of the rat 555
WEST, HAROLD D., NELSON C. JEFFERSON AND RAVEN E. RIVERA. The effect of aromatic hydrocarbons on growth. The reversible inhibition of pantothenic acid by sulfapyridine 471
Wheat bread, peeled, vitamin B complex of 265
—— breads, nutritive value of, contribution of non-fat milk solids to 585
WILDER, RUSSELL M. See Williams, Mason, Cusick and Wilder 361
—— See Williams, Mason and Wilder 71
WILLIAMS, HAROLD H., ELIZABETH T. ENDICOTT, MARION L. SHEPHERD, HARRY GALBRAITH AND ICIE G. MACY. Fat excretion by normal children 379
WILLIAMS, RAY D., HAROLD L. MASON, PAUL L. CUSICK AND RUSSELL M. WILDER. Observations on induced riboflavin deficiency and the riboflavin requirement of man 361
WILLIAM, RAY D., HAROLD L. MASON AND RUSSELL M. WILDER. The minimum daily requirement of thiamine of man 71
WILLIAMS, RAY D. See Higgins, Williams and Mason 229
WINTERS, JET. See Hemphill, Koenig and Winters 285
Women, normal college, basal metabolism of 245
WOODS, ELLA. See Brown, Fincke, Richardson, Todhunter and Woods 411
WRIGHT, LEMUEL D. See Welch and Wright 555

ZMACHINSKY, ANNA. See Ellis, Zmachinsky and Sherman 153

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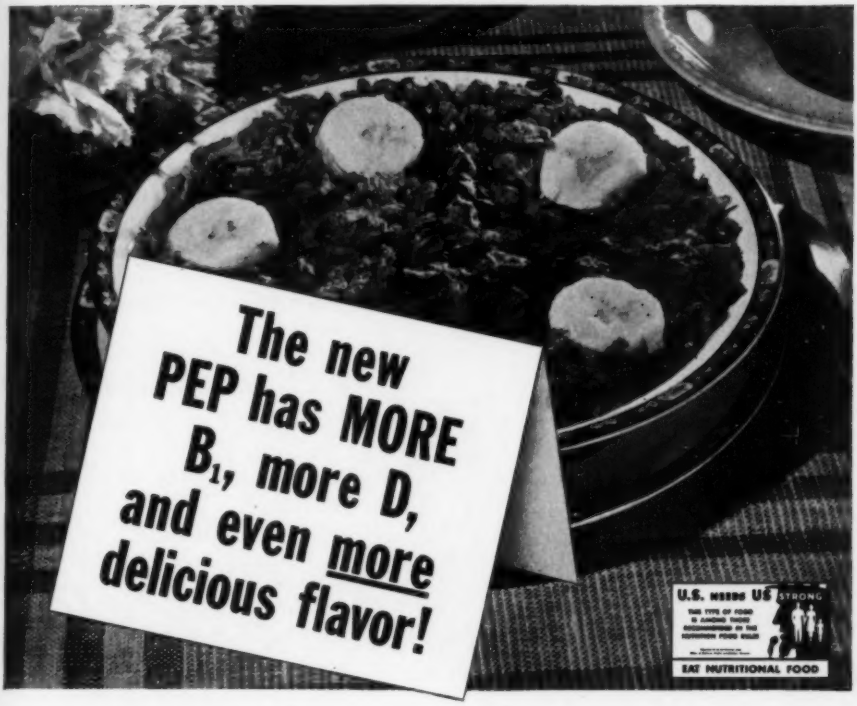

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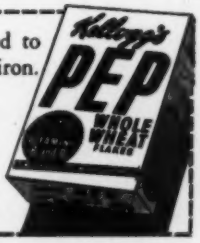

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THE JOURNAL OF NUTRITION

VOL. 25 JUNE 10, 1943 No. 6

CONTENTS

CARROLL F. SHUKERS AND PAUL L. DAY. The effects of inanition and riboflavin deficiency upon the blood picture of the rat 511

DORIS I. ALLEN. The assay of vitamin B_1 in New Zealand materials by the thiochrome method 521

G. H. ELLIS AND K. C. HAMNER. The carotene content of tomatoes as influenced by various factors. One figure 539

A. D. WELCH AND LEMUEL D. WRIGHT. With the technical assistance of K. L. Sprague. The role of "folic acid" and biotin in the nutrition of the rat 555

MIRIAM F. CLARK AND MARIE LECHYCKA. The biological assay of pyridoxine (vitamin B_6). Two figures 571

H. H. MITCHELL, T. S. HAMILTON AND J. B. SHIELDS. With the technical assistance of J. R. Beadles. The contribution of non-fat milk solids to the nutritive value of wheat breads. Three figures 585

ALICE B. ROHRER AND H. C. SHERMAN. The bodily store of vitamin A as influenced by age and by food 605

THE AMERICAN INSTITUTE OF NUTRITION. Résumé of Council meeting 611

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